# ROLE OF IL-17 IN HIV-SPECIFIC CD8$^+$ T CELL IMMUNITY

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**STATEMENT**

I certify that this thesis and the research to which it pertains is an original work except where otherwise acknowledged. Dr.David Boyle provided the parent rFPV and rVV constructs. All the novel HIV-1 vaccines that were used in this study were constructed by Dr. Ron Jackson and some control rFPV vaccine stocks were prepared by Jill Medveczky for the purpose of these studies. Lisa Pavalinovic prepared the rVV vaccine stocks.The material presented in this thesis has not been submitted for a degree in any university.

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## PRESENTATIONS

**TITLE: Role of IL-17 in HIV-specific immunity. Is IL-17 involved in avidity?**

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**ABSTRACT**

It has been more than three decades since HIV (Human Immunodeficiency virus)-1 has been identified as the causative agent of AIDS, but an effective vaccine is still underway. Various vaccine vector delivery systems have been developed to enhance $CD8^+$ T cell mediated immunity against HIV-1. In our laboratory, heterologous mucosal/systemic poxvirus prime-boost immunizations have shown to induce high avidity HIV-specific $CD8^+$ T cell with excellent protective immunity. These studies also revealed that mucosal immunization induced lower numbers of IL-4/IL-13 expressing HIV-specific $CD8^+$ T cells compared to pure systemic delivery. Data indicated that the route of immunization can determine the quality or avidity of $CD8^+$ T cell immunity and this is mainly governed by Th2 cytokines IL-4 and IL-13.

Th17 cells are a newly discovered subset of T cells that specifically produce cytokines IL-17A-F. It is known that these Th17 cells are not only involved in the pathogenesis of autoimmune diseases but also in the protective immunity by recruiting and activating neutrophils. During HIV-1 infection, these Th17 producing $CD4^+$ T cells are depleted and therefore considered to play a role in immunopathogenesis of HIV-1. Various studies have shown that both Th1 and Th2 cytokines negatively regulate IL-17A producing $CD4^+$ T cells in order to mediate their effector immune response. As Ranasinghe *et al.* have shown that IL-4/IL-13 modulate the quality of $CD8^+$ T cells, this study aims to establish whether the expression of IL-17A by HIV-specific $CD8^+$ T cells is dependent on Th2 cytokines IL-4, IL-13 or Th1 cytokine IFN-γ. Wild type BALB/c, IL-4, IL-13 and STAT6 KO mice were prime-boost immunized with control vaccine (FPV-HIV/VV-HIV) and the expression of IL-17A in spleen and lung were evaluated 14 days post booster vaccination. Data indicated that the expression of IL-17A was significantly enhanced in HIV specific $CD8^+$ T cells obtained from KO mice compared to WT BALB/c control mice. But IFN-γ did not have any effect on the IL-17A expression. This data clearly established that the expression of IL-17A in HIV-specific $CD8^+$ T cells was modulated mainly by IL-4 compared to IL-13 (IL-4 >IL-13).

To further investigate these findings and better understand the transcriptional regulation of IL-17A; Wild type BALB/c, IL-4, IL-13 and STAT6 KO mice were prime-boost immunized with control vaccine (FPV-HIV/VV-HIV). RT-PCR was performed to evaluate the IL-17A regulatory factors in $CD8^+$ T cells following HIV-specific

($K^dGag_{197-205}$) peptide stimulation. Data showed that IL-17A, TGF-β, IL-6 and ROR-γt mRNA levels were highly elevated in $CD8^+$ T cells obtained from IL-4 KO mice compared to the other groups tested. This data further confirmed that IL-4 played a predominant role in down regulating IL-17A induction, and TGF-β, IL-6 and ROR-γt not IL-23a were involved in this regulation. Data also confirmed that GATA3 and T-bet did not have any role in regulation of IL-17A expression in $CD8^+$ T cells. Moreover, the granzyme-B expression was lower in IL-4 KO mice compared to IL-13 KO mice, indicating that the IL-17A producing $CD8^+$ T cells in IL-4 KO mice were less cytotoxic compared to the IL-13 KO mice.

As previous studies in our laboratory have shown that IL-13 can significantly modulate the avidity of HIV-specific $CD8^+$ T cells, recently, Ranasinghe *et al.* have developed a vaccine that can temporarily inhibit IL-13 at the vaccination site (FPV-HIV IL-13Rα2/VV-HIV IL-13Rα2) and this vaccine can enhance both the magnitude and avidity of HIV-specific $CD8^+$ T cells compared to the control vaccine (FPV-HIV/VV-HIV). Since, IL-4/IL-13 have shown to modulate IL-17A expression, in this study, the expression of IL-17A in HIV specific $CD8^+$ T cells was also evaluated using control vaccine and novel IL-13 inhibitor vaccine at 3 days, 14 days, 8 weeks and post-challenge. Data indicated that compared to the control vaccine, IL-13 inhibitor vaccine showed enhanced IL-17A expression by HIV-specific $CD8^+$ T cells at effector stage and following surrogate influenza-HIV mucosal challenge in both spleen and lung. The responses following challenge were much greater than at effector stage suggesting that IL-17Aexpression can be considered as an effective marker of protective immunity.

Collectively, the data indicate that more than IL-13, IL-4 regulates the IL-7A expression in HIV-specific $CD8^+$ T cells. As IL-4 and IL-13 are involved in regulating the avidity of CTLs, IL-17A also plays an indirect role in modulating $CD8^+$ T cell avidity and protective immunity.

## LIST OF ABBREVIATIONS

| | |
|---|---|
| Ab | Antibody |
| Ag | Antigen |
| AIDS | Acquired Immunodeficiency Syndrome |
| APC | Antigen Presenting Cell |
| BCR | B Cell receptor |
| BFA | Brefeldin A |
| CCL2 | Chemokine C-C motif Ligand-2 |
| CD | Cluster of Differentiation |
| cDNA | Complimentary Deoxyribonucleic Acid |
| CMI | Cell Mediated Immunity |
| CpG | Cytosine phosphate Guanosine |
| CTL | Cytotoxic T Lymphocyte |
| DC | Dendritic Cell |
| DKO | Double gene knock out |
| DNA | Deoxyribonucleic Acid |
| DMSO | Dimethyl Sulphoxide |
| dsRNA | double stranded Ribonucleic acid |
| Eomes | Eomesodermin |
| ELIspot | Enzyme Linked ImmunoSPOT |
| FACS | Fluorescence Activated Cell Sorter |
| FCS | Fetal Calf Serum |
| FITC | Fluorescein-isothiocyanate |
| Foxp3 | Fork-head box transcription factor-3 |
| FPV | Fowl Pox Virus |
| GM-CSF | Granulocyte Mcrophage Colony Stimulating Factor |
| GALT | Gut Associated Lymphoid Tissue |
| γδ | gamma delta |
| HEPES | 4-(2-Hydroxyethyl)-1 piperazine-ethanesulfonic acid |
| HIV | Human Immunodeficiency Virus |
| ICS | Intracellular Cytokine Staining |
| IC-FIX | Intracellular fixation buffer |
| IFN-γ | Interferon gamma |

| | |
|---|---|
| IL | Interleukin |
| IL-4Rα | IL-4 receptor alpha |
| IL-13Rα2 | IL-13 receptor alpha 2 |
| i.m | Intramuscular |
| i.n | Intranasal |
| IRF | Interferon Regulatory factor |
| IU | International Units |
| i.v | Intravenous |
| JAK | Janus Kinase |
| JCSMR | John Curtin School of Medical Research |
| kDa | kilo Dalton |
| KO | Gene Knock out |
| LCMV | Lymphocytic Choriomeningitis Virus |
| LC | Langerhans cells |
| LTi | lymphoid-tissue inducer |
| MCMV | Murine Cytomegalo Virus |
| MEM | Minimal Essesntial Medium |
| MHC | Major Histocompatibility Complex |
| MOI | Multiplicity of Infection |
| mRNA | Messenger Ribonucleic Acid |
| MVA | Modified Vaccinia Ankara |
| NF-κβ | Nuclear Factor Kappa- B |
| NK | Natural Killer |
| OVA | Ovalbumin |
| PAMP | Pathogen Associated Molecular Patterns |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| PE | Phycoerythrin |
| PFU | Plaque Forming Units |
| PRRs | Patteren Recognition Receptors |
| rVV | recombinant Vaccinia Virus |
| ROR | Retinoic acid receptor related Orphan Receptor |
| rpm | Rotations per minute |
| RPMI-1640 | Roswell ark-Memorial Institute-1640 cell culture medium |
| RT | Room Temperature |

| | |
|---|---|
| RT-PCR | Reverse Transcription Polymerase Chain Reaction |
| SEF | Similar Expression of Fibroblast growth factor genes |
| SFU | Spot forming Units |
| SIV | Simian Immunodeficiency Virus |
| STAT1 | Signal Transducer and Activation of Transcription-1 |
| TAE | Tris Acetate EDTA |
| TAK1 | TGF-β activated kinase |
| T-bet | T-box transcription factor |
| Tc | T cytotoxic cell |
| TCR | T cell Receptor |
| TGF-β | Transformation Growth Factor-Beta |
| TK | Thymidine Kinase |
| Th | T helper |
| TNF | Tumor Necrosis Factor |
| TRAF-6 | TNF-receptor associate factor-6 |
| TLR | Toll Like receptor |
| TNF | Tumor Necrosis Factor |
| VV | Vaccinia Virus |

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# CHAPTER 1: Introduction

## 1.1 The Immune system

The immune system comprises many cells and organs that collectively work together interdependently to induce immunity against pathogens. The key function of the immune system is based on its ability to distinguish "self" from "non-self" antigens [1]. Once the foreign or non-self microorganism is identified, series of immune reactions occur to evade the pathogen. The immune system is generally divided into two arms. They are 1) the innate immunity, which is non-specific and 2) the adaptive immunity, which is specific.

### 1.1.1 Innate and Adaptive immunity

The cells of the innate immune system provide a wider range of protection against non-specific pathogens. Innate immune system comprises of a physical barrier, complement system and a chemical or cellular barrier. The cellular mediators of the innate immune system include neutrophils, macrophages, Dendritic Cells (DCs) and Natural Killer (NK) cells. Upon encountering an antigen (Ag), these innate immune cells express germ line encoded receptors called pathogen recognition receptors (PRRs), which provide an immediate response against the antigen. These receptors identify a unique set of structures common to all the pathogens called pathogen associated molecular patterns (PAMPs). PAMPs include a wide variety of structures ranging from all components of microbial membranes, cell walls, unmethylated Cytosine phosphate Guanosine (CpG) motifs, double stranded Ribonucleic acid (dsRNA), mannans and reserved structures of cell wall. Among the classes of PRRs is the special type of structures called as Toll Like receptors (TLRs). The recognition of these TLRs make the innate immune cells capable of discriminating self and non-self Ag [2] (Fig 1.1). Indeed, these TLRs also act as an adjuvant receptors that bridge the innate and the adaptive immune system [3].

The adaptive immune response protects the host by providing specific, diverse and long lasting immunity through immunological memory on subsequent antigenic challenge. The adaptive immune response functions through humoral and Cell Mediated Immunity (CMI) [4], [1]. Modulation of different types of adaptive immune response depends on various pathways that the DCs mature and present the Ag to the specific cells. Humoral immunity refers to the antibody (Ab) mediated immunity against the extracellular pathogen. It includes all non-cellular components of the blood such as plasma cells and lymphatic fluid. The activation of humoral immune response is initiated by the

recognition of Ags by the B cells via immunoglobulins (Ig) or B Cell Receptor (BCR), which processes and presents the antigen on Major Histocompatibility Complex (MHC) class II [5]. On the other hand, CMI involves activation of certain subsets of cells against the intracellular pathogen such as virus and intracellular bacteria. CMI is mediated through $CD8^+$ or $CD4^+$ T lymphocytes, which recognize the antigenic peptide in the form of MHC, class I ($CD8^+$ T cells) or MHC class II peptides ($CD4^+$ T cells) through T cell receptor (TCR). This recognition further activates B cells and T cells to kill the virally infected target cells. The activation of T cells require two signals i.e. 1) T cell receptor (TCR) ligation and 2) simultaneous engagement of CD28 on T cells by CD80 and CD86 on the APC [6, 7] (Fig 1.1). These activated T cells become either cytotoxic $CD8^+$ T cells (CTL) and $CD4^+$ T cells which are involved in various effector functions of CMI.

### 1.1.2 Immunological memory

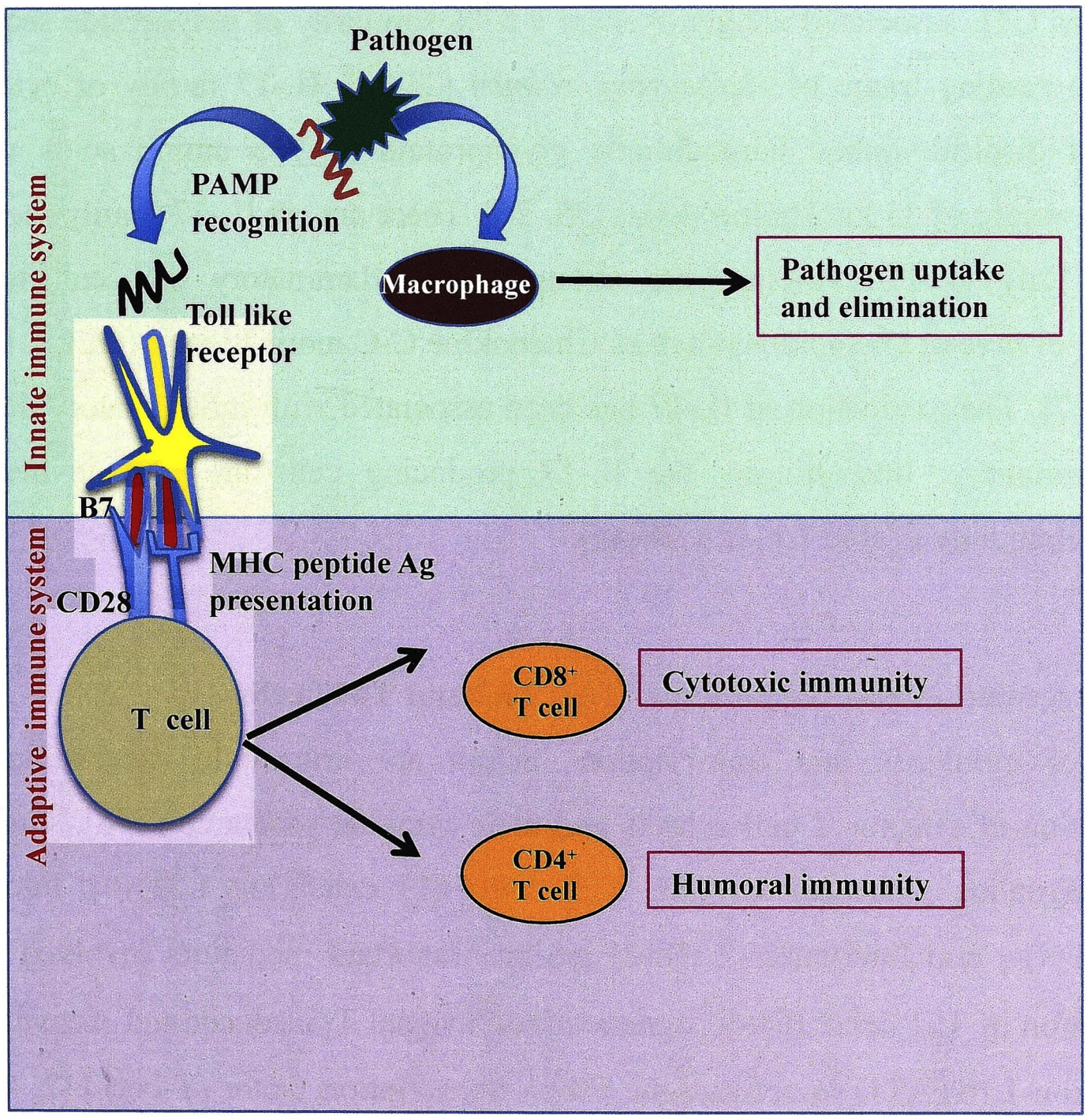
The most important aspect of the adaptive immune response is establishing a state of immunological memory, which plays a major role in protecting the host from reinfection. Thus, obtaining immunological memory becomes a major goal in various vaccine strategies. Both memory B and T cells are involved in generating long-term immunological memory. Memory B cells, in response to secondary antigenic challenge proliferate and differentiate into plasma cells thereby secreting antibody [8-10]. The antibodies produced by the plasma cells in primary and early secondary response are crucial in driving the B cell maturation. Unlike the B cells, memory T cells are diverse in their function and phenotype. T cells are divided into naïve, effector and memory cells depending on the antigenic challenge. Naïve T cells reside in the body before encountering of the Ag. Upon antigenic challenge, these naïve T cells become activated and transformed into effector cell to clear the infection. Once the infection has been cleared, Some of these T cells reside in the body as memory lymphocytes to protect the host from the same antigenic challenge [11].

## 1.2. Th17 cells

Upon TCR mediated activation, naïve $CD4^+$ T cells can differentiate into four major subsets including Th1, Th2, Th17, and Treg, which mediate the immune response. Treg cells also called as suppressor cells provide tolerance to self-Ag thereby preventing autoimmunity. Th1 and Th2 mediate cellular and humoral immunity by their cytokine

**Fig 1.1 Innate and adaptive immunity**

*Innate immunity: Antigen presenting cell such as dendritic cell, macrophages recognize antigen (micro-organisms) by TLR (Toll like receptor) through PAMPs, which results in non-specific or innate immune response. Adaptive immunity: T cells ($CD8^+$ and $CD4^+$ T cells) recognize antigen in the form of MHC I and II peptides to mediate cytotoxic or humoral immunity.*


Pathogen
PAMP
recognition
Innate immune system
Macrophage
Pathogen uptake
and elimination
Toll like
receptor
B7
MHC peptide Ag
presentation
CD28
Adaptive immune system
T cell
CD8+
T cell
Cytotoxic immunity
CD4+
T cell
Humoral immunity

profiles [12, 13]. In addition to Th1 and Th2 subsets, Th17 cells are the newly discovered effector $CD4^+$ T cell subsets which specifically produce Interleukin (IL)-17A-F (Fig 1.2) [14]. These IL-17A producing cells mediate both innate and adaptive arms of the immune response and play a vital role in tissue surveillance especially in the gut, lung and skin by inducing early neutrophil recruitment [15]. The main sources of innate IL-17 producing cells are NK cells, gamma delta (γδ) -T cells, lymphoid-tissue inducer -like cells and myeloid cells [16-18]. In the context of the adaptive immune system, $CD4^+$ and $CD8^+$ T cells are the producers of IL-17A [19-21]. IL-17, also described as CTL associated antigen-8 share a 57% similarity of polypeptide sequence to an open reading frame of *Herpesvirus saimiri* [22-24]. IL-17 family of cytokines consists of disulfide-linked homodimeric glycoprotein of 155 amino acids with a molecular weight of 35 kiloDalton (kDa) [25, 26]. There are six IL-17 family members IL-17A-F [24]. IL-17 cytokines are strongly pro-inflammatory and enhance the expression of several chemokines such as Chemokine C-C motif Ligand (CCL2, CCL7, CCL20) [27]. The production of IL-17 has been associated with inflammatory diseases and autoimmunity. Interestingly, the IL-17 producing cells are also involved in protective immunity (Table 1.1) [21, 28-44].

### 1.2.1 Differentiation and regulation of Th1, Th2 and Th17 cells

Network of cytokines and transcription factors are crucial in determining the differentiation of effector T cell subsets and their cytokine production [45]. There are different signaling pathways for Th1, Th2 and Th17 cells (Fig 1.3)[46]. Interferon gamma (IFN)-γ and Interleukin-2 (IL-2) are the important mediators involved in the differentiation of Th1 cells. IFN-γ, signals through signal Transducer and Activation of Transcription-1 (STAT1) to activate the T-box transcription factor (T-bet) [47, 48]. T-bet induces IL-12 Receptor (R) β2 subunit which together with IL-12Rβ1 form IL-12R complex. IL-12 mediates activation of STAT4 through IL-12R complex and stabilizes IFN-γ production.

On the other hand, Th2 cells require TCR with IL-4 mediated signaling to induce STAT6 transcription, thereby promoting the transcription of GATA3 [49]. GATA3 then promotes IL-4 production for the development of Th2 cells. In contrast to Th1 and Th2 subsets requiring IFN-γ and IL-4 cytokines respectively, Th17 cells do not require IL-17 for its differentiation. It is established that Th17 differentiation requires IL-6 and

**Table 1.1**

| Pathogen | Vaccine | Organ | Mechanism/Reference |
|---|---|---|---|
| M. tuberculosis | 1) Peptide vaccine | Lung | Recruitment of CD4 Th1 cells (21) |
| | 2) Subunit vaccine | Lung | (28) |
| | 3) Viral vectors expressing protective antigen | Lung | (40) |
| | 4) Primary BCG vaccination followed by booster with viral vectors expressing protective antigens | Lung | (44) |
| | 5) Primary BCG vaccination followed by booster with DNA vaccine encoding protective antigens | | (39) |
| | 6) DNA vaccine expressing protective antigen and | Lung | (36) |

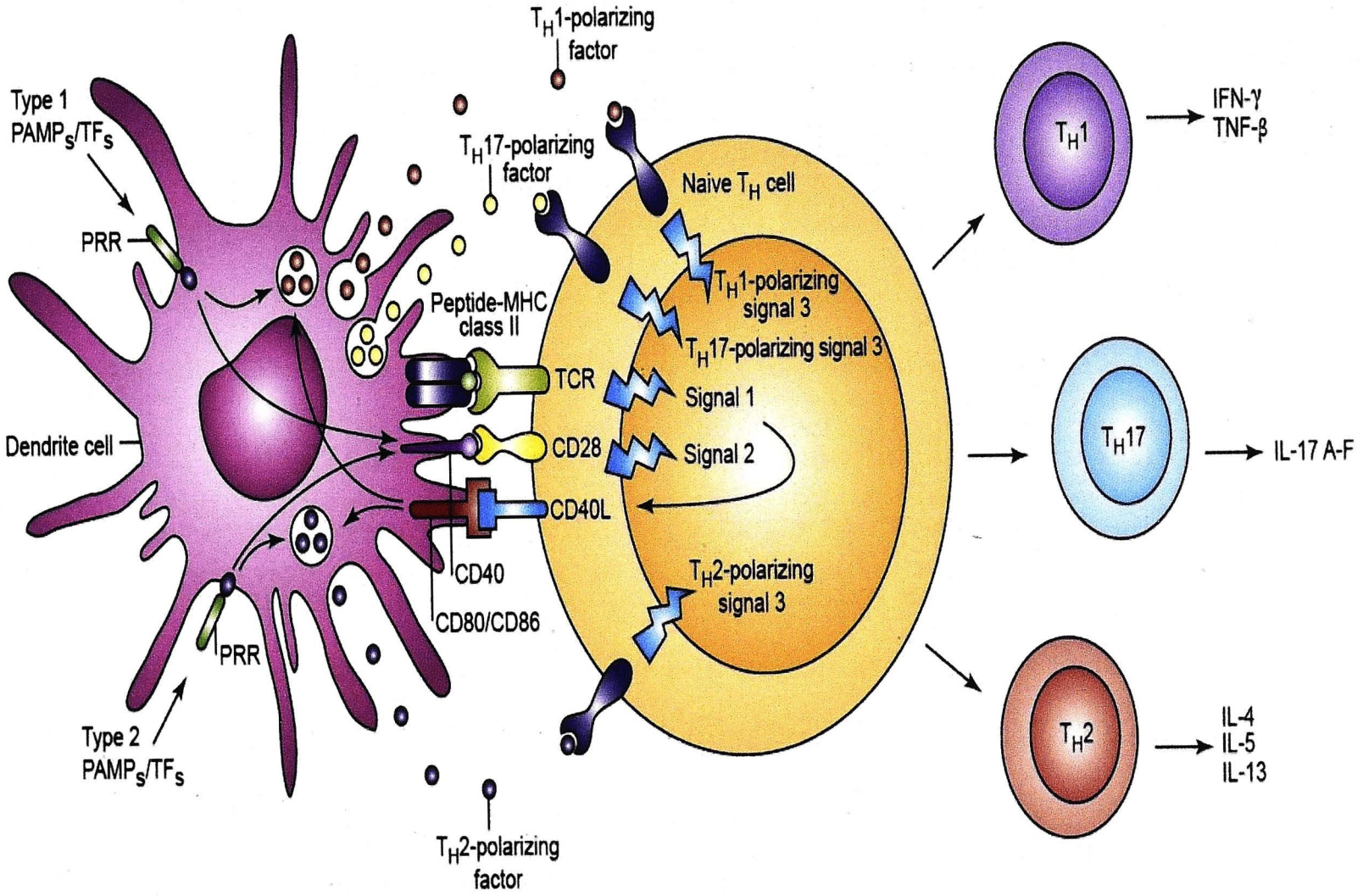
| | | | |
|---|---|---|---|
| | expressing IL-23 | | |
| S. pneumoniae | Pneumococcal whole-cell antigen | Lung | (33)<br>Monocyte, macrophage, and neutrophil recruitment |
| | Cell Wall polysaccharides | Lung | and phagocytic killing (35,29) |
| | Live organism | Lung | Monocyte, macrophage, and neutophil recruitment (30) |
| B. pertussis | Whole cell vaccines | Lung | Neutrophil recruitment and enhanced phagocytic killing (42) |
| H. pylori | H. pylori lysate and | Recruitment of neutrophils, | killing activity (34) |

| | | | |
|---|---|---|---|
| | recombinant urease<br>Gut | monocytes and macrophages<br>and enhanced | |
| P. aeruginosa | Live organism | Lung | Recruitment of neutrophils and increased bacterial clearance (32) |
| Rhesus rotavirus | Recombinant antigen | Intestine | (37,38) |
| Influenza Virus | DNA Vaccine expressing protective antigen and IL-23 | Lung | (43,31) |

Table reproduced from Lin et al. 2010 (41)

**Fig 1.2: Th1 and Th2 and Th17 immune response**

*Naïve $CD4^+$ T cell through its activation by TCR and antigen presenting cell derived co-stimulatory molecules differentiate into different Th subsets, such as Th1 secreting IFN-γ, IL-2 and Th2 secreting IL-4, IL-13, IL-10 and Th17 secreting IL-17 A-F. This differentiation of T cell subsets leads to various effector functions in clearing the infection.*


$T_H1$-polarizing factor
Type 1 PAMPs/TFs
$T_H17$-polarizing factor
Naive $T_H$ cell
PRR
$T_H1$-polarizing signal 3
Peptide-MHC class II
$T_H17$-polarizing signal 3
TCR
Signal 1
Dendrite cell
CD28
Signal 2
CD40L
CD40
$T_H2$-polarizing signal 3
CD80/CD86
PRR
Type 2 PAMPs/TFs
$T_H2$-polarizing factor
$T_H1$
IFN-γ
TNF-β
$T_H17$
IL-17 A-F
$T_H2$
IL-4
IL-5
IL-13

Transformation Growth Factor-Beta (TGF-β) initially for its development [50]. Using IL-6 and TGF-β transgenic mice, the essential role of these cytokines has been illustrated in various studies [51-53]. IL-6, a strong pro-inflammatory cytokine is expressed by the cells of the innate immune system on its interaction with specific PRRs. TGF-β being an anti-inflammatory cytokine, through its downstream signaling with IL-6 receptors is involved in inducing IL-17 in naïve T cells [54]. TGF-β is mainly involved in inducing Treg transcription factor Fork-head box transcription factor-3 (Foxp3) for the activation and maintenance of Treg cells in the peripheral immune compartment [55, 56]. Thus, two cytokines (IL-6 and TGF-β) with their opposing effects interact with each other to drive the differentiation of Th17 cells. The cytokine promoters such as IL-6, IL-21 and IL-23 activates STAT3, which then binds to IL-17 and IL-21 to further activate Retinoic acid receptor related orphan receptor-γt (ROR-γt), inducing the differentiation of Th17 [57-59]. Studies have also shown that interferon regulatory factor-4 (IRF-4) is also required in the earlier stages of Th17 development [60, 61].

### 1.2.2 Cross inhibition of Th1, Th2, Treg and Th17 cells

The differentiation of effector Th subsets involves the cross inhibition of other subset and their effector cytokines production [62]. For example, the transcription factor T-bet regulates IFN-γ production and inhibits the expression of Th2 cytokine by suppressing the GATA3 and IL-4 effector functions [63]. In contrast, over expression of GATA3 also suppresses T-bet and its effector cytokine production. Similarly, FoxP3 (transcription factor for Treg cells) and ROR-γt (transcription factor for Th17 cells) suppress each other during their cytokine differentiation [64, 65]. Indeed, studies have shown that inhibiting ROR-γt mediated IL-17 increased the expression FoxP3 [66]. In addition, IFN-γ, IL-4, IL-13 through their transcription factors T-bet and GATA3 respectively suppress ROR-γt resulting in the inhibition of IL-17A production [20, 67] (Fig 1.3). Recent studies have also shown that IFN-γ deficient mice produce increased numbers of IL-17 producing T cells during mycobacterial infection [68]. Furthermore, epicutaneous sensitization using IL-13, IL-4 KO (Gene knock out) and IL-13 IL-4 DKO (Double gene knock out) mice also showed exaggerated IL-17A production in the lung resulting in airway inflammation [69]. In a recent study, Newcomb *et al.* have shown that a functional IL-13R is expressed on $CD4^{+}$Th17 cells and IL-13 negatively regulates IL-17 production by down-regulating ROR-γt while increasing STAT6 and GATA3

expression [70, 71]. These studies demonstrate that Th2 and Th1 cytokines and their transcription factors modulate the function of IL-17 production by T cells. This forms the basis of the current project.

### 1.2.3 IL-17 family

IL-17 includes a family of cytokines IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (also called as IL-25) and IL-17F [24] (Table 1.2, Fig 1.4)[72]. IL-17A and F share a 55 % similarity and are produced by a variety of cell types including $CD4^+$, $CD8^+$, NK cells, γδ-T cells and also neutrophils [73, 74]. IL-17A and F are the key cytokines known for activating and recruiting neutrophils to the site of infection [75]. Both IL-17A and F have been shown to mediate autoimmunity and inflammation. IL-17B and C have been shown to be involved in the transcription of pro-inflammatory genes similar to that of IL-17A and F [76]. Also IL-17D is involved in pro-inflammatory gene expression profiles in endothelial cells [77]. In contrast, IL-17E (IL-25) is produced during Th2 differentiation to induce chemokines such as RANTES and Eotaxin-1 against allergic response [78]. IL-25 has also shown to play a protective role in nematode infection by recruiting Th2 cytokines (IL-4/IL-5/IL-13) to resolve the infection and also induce allergic response in asthma models [79-81]. IL-17E also inhibits Th17 cell development through the induction of IL-13 by dendritic cells [82] (Table 1.2) [77].

### 1.2.4 Signaling of IL-17

It has been shown that IL-17 family cytokines signal through the receptors of the IL-17R family. The IL-17R family consists of IL-17RA, IL-17RB, IL-17RC, IL-17RD and IL-17RE. All of these receptors are transmembrane domain containing 499-866 amino acids [77]. There is no receptor for IL-17F as IL-17A and F share a common receptor IL-17RA, with IL-17A of higher affinity. When binding IL-17A or IL-17F, the IL-17RA processes a conformational change resulting in the dissociation of its intracellular domain. IL-17RA and IL-17RC have been involved in the binding of IL-17A and IL-17F. IL-17RB binds to IL-17B and IL-17E (also called as IL-25) and the subunits of these receptors are highly expressed in endothelial cells and Th2 cells. IL-17E also activates NFATc1 (Nuclear factor of activated T cells, cytoplasmic1) resulting in enhanced IL-4 expression by Th2 cells [83]. IL-17RD is involved in the proinflammatory activities and its receptors is unknown (Table 1.2). In mammals, IL-17 exists as the closest homolog to Similar Expression of Fibroblast growth factor genes

**Fig.1.3 Regulation of Th subsets**

*Differentiation of Th1, Th2 and Th17 cells: IFN-γ receptor signals STAT1 (Signal transducer and activation of transcription) resulting in the activation of T-Bet transcription factor to induce Th1 effector response. In contrast, IL-4 receptor signals through STAT6, which promote GATA-3 transcription to induce the differentiation of Th2 cells. iTreg differentiate through the activation of Fox-p3 signal by the secretion of TGF-β. The differentiation of Th17 initially requires cytokine promoters IL-6, IL-21 and IL-23 to activate STAT3 to induce IL-21 and IL-23R for Th17 induction. Finally, ROR-γt is required for its expansion and survival. Fig modified from Yu et al.2008 [46].*

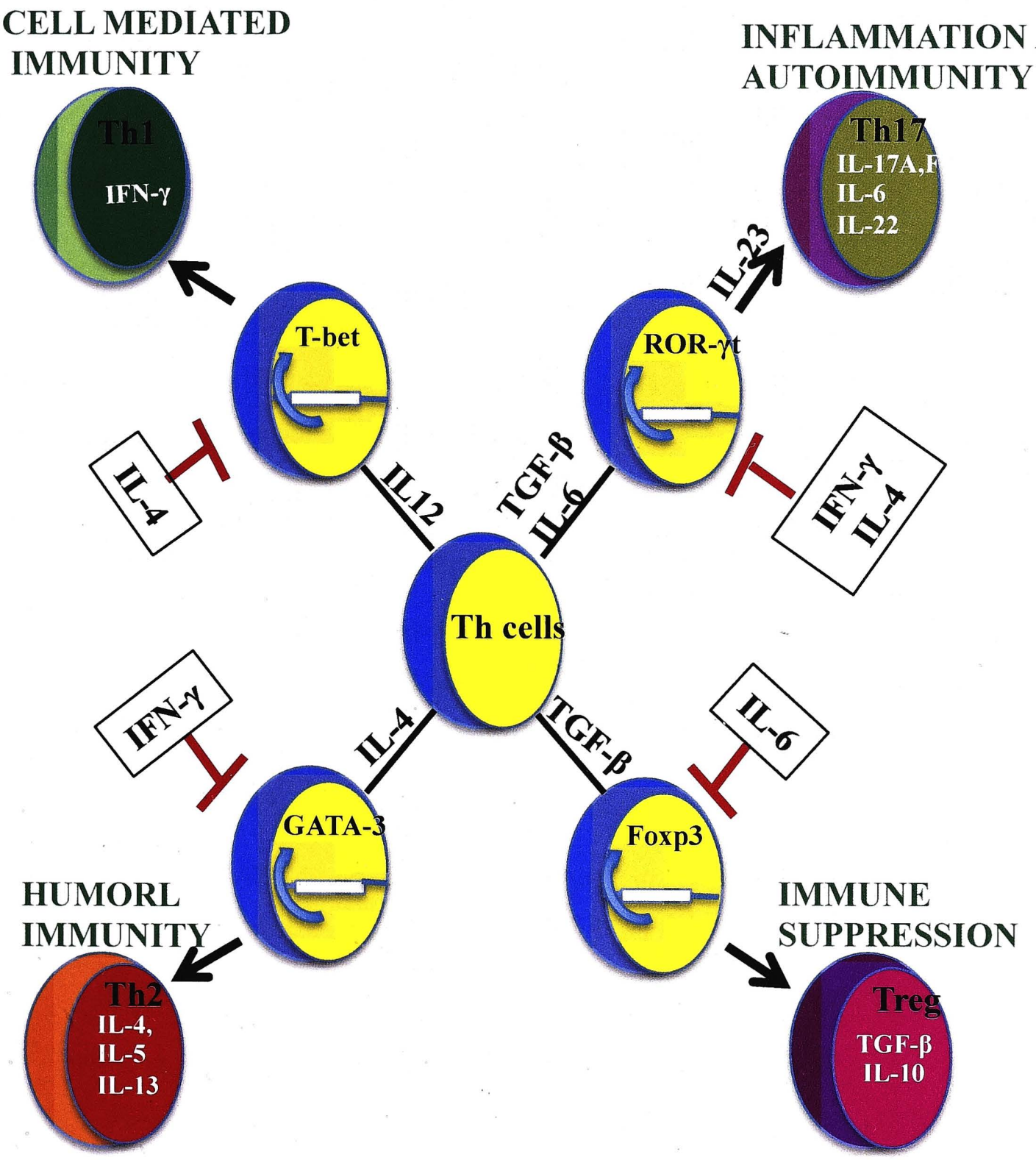
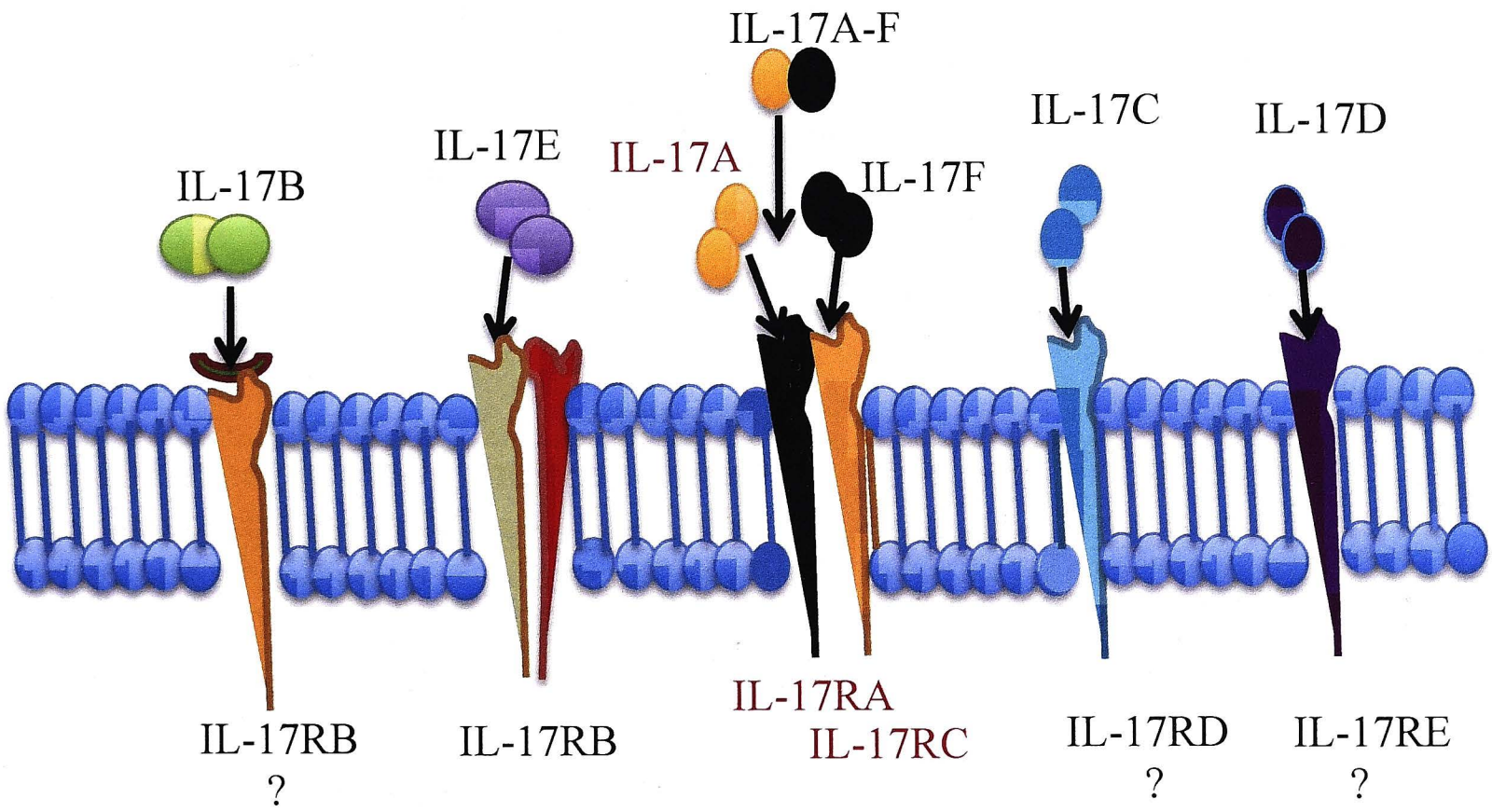

CELL MEDIATED IMMUNITY
Th1
IFN-γ
INFLAMMATION AND AUTOIMMUNITY
Th17
IL-17A,F
IL-6
IL-22
IL-23
T-bet
ROR-γt
IL-4
IL12
TGF-β
IL-6
IFN-γ
IL-4
Th cells
IFN-γ
IL-4
TGF-β
IL-6
GATA-3
Foxp3
HUMORL IMMUNITY
IMMUNE SUPPRESSION
Th2
IL-4,
IL-5
IL-13
Treg
TGF-β
IL-10

**Fig 1.4: IL-17 family-ligand receptors (IL-17R)**

*IL-17R family contains IL-17RA, IL-17RB, IL-17RC, IL-17RD and IL-17 RE, which are single transmembrane domain bound receptors consisting 499-866 amino acids. These various receptor complexes mediate with specific ligands to induce the IL-17A signaling. Figure modified from Zepp Wu et al. 2011 [72].*




**Autoimmunity,**
**inflammation**
**and**
**HOST DEFENCE**

**Table 1.2: IL-17 family, receptors and functions**

| Family member | Receptors | Functions | Expression |
|---|---|---|---|
| IL-17A | IL-17RA and IL-17RC | Autoimmunity and neutrophil recruitment, protective immunity | Th17 cells, $CD8^+$ T cells, NK cells,γδ T cells |
| IL-17B | IL-17RB | Proinflammatory activites | Cells of gastrointestinal tract and pancreas |
| IL-17C | IL-17RE | Proinflammatory activites | Kidney cells and cells of prostate |
| IL-17D | --- | Proinflammatory activites | Cells of lung, muscles, brain, heart, pancreas |
| IL-17E | IL-17RA and IL-17RB | Induces Th2 responses and suppresses Th17 cell responses | Intraepithelial lymphocytes,eosinophils, basophils, NKT cells,Th2 cells, mast cells |
| IL-17F | IL-17RA and IL-17RC | Neutrophil recruitment and immunity to extracellular pathogens | Th17 cells, $CD8^+$ T cells, NK cells,γδ T cells |

*Table adapted from Gaffen et al, 2009 [77]*

(SEF), which acts in inhibiting fibroblast growth factor signaling. The sequence homology in the cytoplasmic region of IL-17 family of receptors are also called as SEFIR residing in SEF and IL-17Rs domain [72]. Also, adaptor protein Act-1 plays a key role in the signaling of IL-17R complex [84]. Act-1 signals IL-17A and IL-17F via IL-17RA and IL-17RC through SEFIR-SEFIR interactions leading to the recruitment of TNF-receptor associate factor-6 (TRAF-6) and TGF-β activated kinase (TAK1). The series of signaling results in the downstream signaling/activation of Nuclear Factor-κβ (NF-κβ) / Mitogen-activated protein kinase (MAPK) pathways [85] (Fig 1.5). In addition, IL-17 has also shown to be involved in the activation Janus Kinase (JAK)-1, JAK-2 associated phosphoinositide-3 kinase pathway which act with NF-κβ in enhancing gene expression of host defense [86].

## 1.3 Vaccines

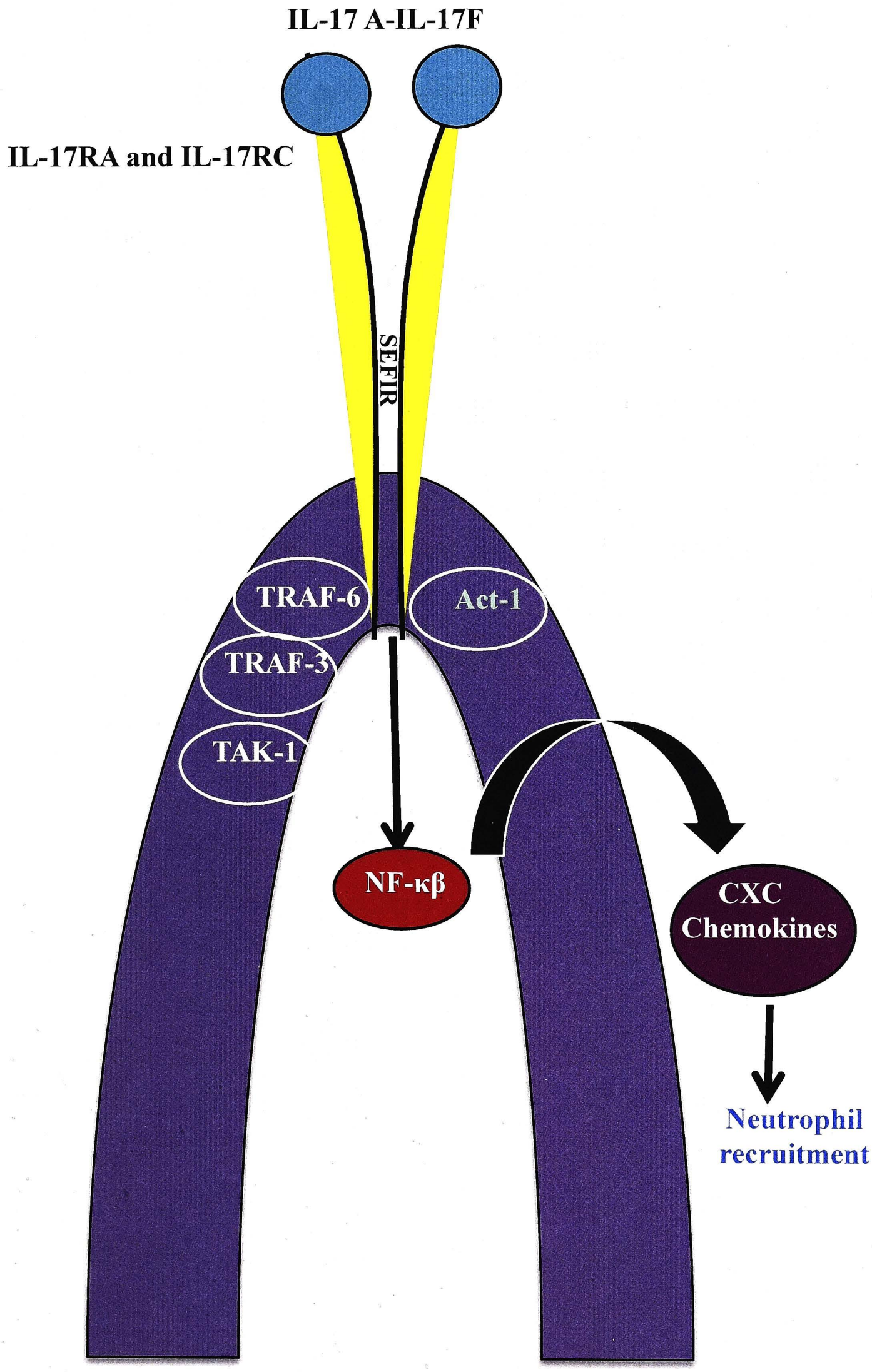
Vaccines have played a crucial role in protecting the humans against deadly diseases [87]. The successful application of vaccinia virus to protect against smallpox by Edward Jenner represents the beginning of the vaccine era. In general, vaccines mainly function by mimicking the infections and providing long lasting memory response that can potentially protect the vaccine recipient against subsequent antigenic challenge. Ideally, an effective vaccine should provide strong neutralizing antibody responses and T cell mediated immunity against the pathogen. The developments of effective vaccines against infectious diseases have improved the quality of human life. For example, live attenuated viruses, "killed viruses", subunit and recombinant viruses are used in vaccines and were shown to be inducing strong antibody and cell mediated immunity against viruses such as polio, small pox [88], hepatitis B, influenza, mumps, measles and rubella [89-91]. The development of the recent cervical cancer vaccine has been a great success [92]. Despite major advances in vaccine development, there are still no effective vaccines offering protective immunity against many chronic infections such as malaria, Tuberculosis and HIV-1 (Human Immunodeficiency Virus-1).

### 1.3.1 HIV-1 vaccines- current status

Since the discovery of HIV in 1983 [93], HIV-1 has become one of the most devastating diseases especially in sub-Saharan Africa. Today, HIV-1 has claimed over 33 million deaths worldwide. Three decades have passed and still there is no cure for

**Fig.1.5: Receptor signaling of IL-17A and IL-17F**

*Upon activation through the signals, Act-1 protein interact with SEFIR domain of IL-17R complex to activate the transcription factors. This series of signaling leads to downstream signaling /activation of NF-κβ to recruit neutrophils. (Fig modified from Zepp Wu et al. 2011 [72].*


IL-17 A-IL-17F
IL-17RA and IL-17RC
SEFIR
TRAF-6
Act-1
TRAF-3
TAK-1
NF-κβ
CXC
Chemokines
Neutrophil
recruitment

HIV-1 other than life-long anti-retroviral treatment that can reduce the viral load. The main hurdles in developing HIV-1 vaccine are high HIV-1 mutation rates, inability to produce broadly neutralizing antibodies and lack of immune correlates of protection in humans [94, 95]. Many systemic HIV-1 vaccine strategies that have elicited great promise in animal models have failed to induce good protective immunity in human clinical trials. For example, the Merck STEP HIV-1 recombinant adenoviral vaccine trial conducted in 2007 was unexpectedly terminated due to the failure in inducing protection in humans [96].

Therefore, there is a great need to understand why these vaccines are failing in humans, and evaluate the correlates of protective immunity in humans. In the context of effective HIV-1 vaccines, $CD8^+$ T cell mediated immunity and neutralizing antibodies are thought to be important in protective immunity [4, 97-101]. Due to the difficulty in obtaining immunogens to induce high titer neutralizing antibodies with broad specificities, lots of attention has been focused on stimulating HIV-specific T cell responses for their ability to better control the viral replication and its association with long term survival [102, 103].

The development of recombinant DNA (rDNA) immunization in early 1990s offered hope for many diseases for which traditional vaccine delivery approaches were ineffective. These vaccines showed significant advantages over other immunization strategies mainly due to vectors being non-replicative, non-infectious, non-integrating, stable and ease of prepare and their lower cost. Fuller *et al.* were the 1st to demonstrate that gene gun delivery of rDNA expressing envelope glycoprotein - gp120 was able to induce both humoral and cellular mediated immunity in small animal models [104, 105]. However, compared to gene gun delivery single or multiple intra muscular (i.m.), or intravenous (i.v.) rDNA delivery strategies were unable to generate comparable immune outcomes against highly pathogenic organisms such as HIV-1. Since then rDNA vaccines soon became an excellent priming modality in prime boost vaccination [106, 107] (Fig 1.6). As boosting vectors, a range of viral vectors that have been used for vaccination against HIV-1 include avian poxviruses such as Fowl pox virus (Coupar et al., 2006), mammalian poxvirus vectors for example NewYork Vaccinia virus (NYVAC) [108, 109], Modified Vaccinia Ankara (MVA) vectors [110]. Similarly, in the recent years, adenovirus [96, 111, 112], influenza virus [113] and polio virus [114] have also been trialed as vaccine vectors.

In mice and macaques, systemic rDNA priming and recombinant rFPV boost immunizations with HIV-1 antigens have elicited high levels of antigen specific T cell immunity [115-120]. Similarly, Amara *et al.* have shown that HIV-DNA prime followed by MVA-HIV boost immunization generated protective immunity in macaques [121]. Despite the success in animal models most of these DNA/viral prime-boost vaccines have yielded poor outcomes in systemic phase I clinical trials [122, 123]. Alternatively, viral/protein, viral/viral prime-boost immunization strategies were also employed to overcome the poor outcomes with these rDNA vaccines. For example, studies in our laboratory have shown that compared to DNA/viral prime-boost immunizations pox viral/poxviral prime-boost immunization can generate excellent high quality or avidity HIV-specific $CD8^+$ T cell responses [124]. Also, in a recent HIV-1 human clinical trial, a recombinant canary pox vector (ALVAC-HIV) prime followed by recombinant gp120 subunit vaccine (AIDSVAX B/E) booster immunization resulted partial protection against HIV-1 (vaccine efficacy of 31.2%) [125]. Interestingly, these vaccines were not successful at inducing good T or B cell immunity when used as single modality vaccines. Thus, viral/viral or viral/protein prime-boost immunization strategy offers renewed hope for a successful future HIV-1 vaccine.

### 1.3.3 Importance of mucosal immunity and route of immunization

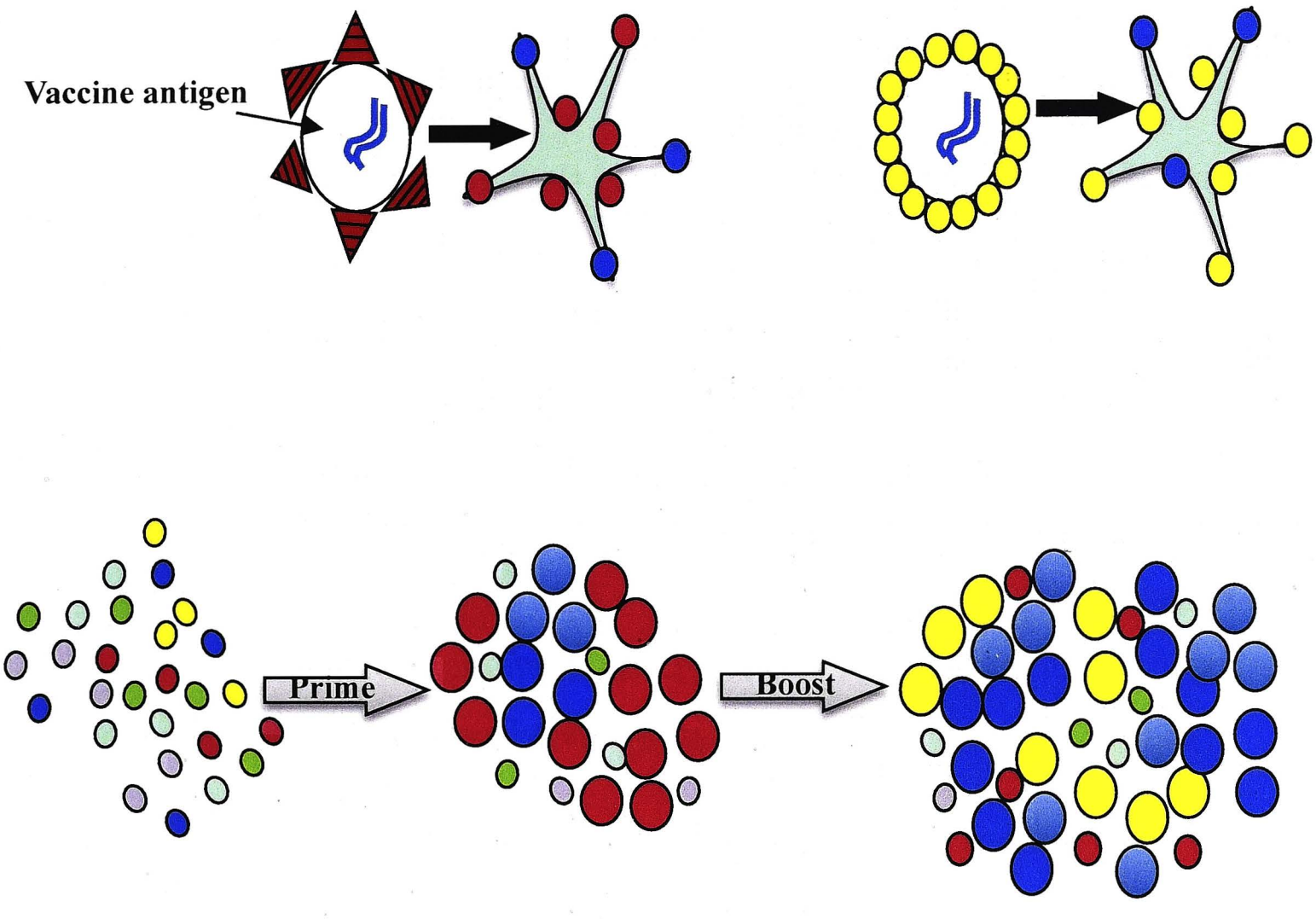
Although the systemic prime-boost vaccination strategies offer strong T cell response, these vaccines have failed to induce protective immunity at the mucosae (i.e. genito-rectal mucosa). Indeed, HIV-1 is firstly encountered at the mucosae and the first $CD4^+$ T cell depletion and viral replication occurs predominantly in the gut mucosae (Fig 1.7) [126-128]. Therefore, the expression of strong sustained antiviral CTL responses at these mucosal sites is thought to be essential for protective immunity against HIV-1. Various studies have demonstrated that mucosal delivery of a vaccine can lead to the generation of immunity at both the local and distant mucosae [129, 130], [131]. Studies from our laboratory have shown that following pure systemic prime-boost immunization FPV-HIV/VV-HIV immunization can induce immunity in systemic or blood compartment but not long lived immunity in the mucosal compartment [132]. This is mainly due to systemic vaccination inducing lower levels of mucosal homing markers ($\alpha4\beta7$ and CCR9) on HIV-specific $CD8^+$ T cells that could migrate to the mucosae [133-135]. Also, studies by Belyakov *et al.* have demonstrated that mucosal administration of either peptide or recombinant HIV vaccine can induce strong CTL in

**Fig 1.6: Prime boost Immunization strategy**

*Same vaccine antigens are encoded in two different vaccine vectors (i,e rFPV and rVV). Following prime initial T cell subset is induced against the encoded antigen and during booster immunizations, these T cell responses are expanded.*

**PRIME VACCINATION**

**BOOST VACCINATION**

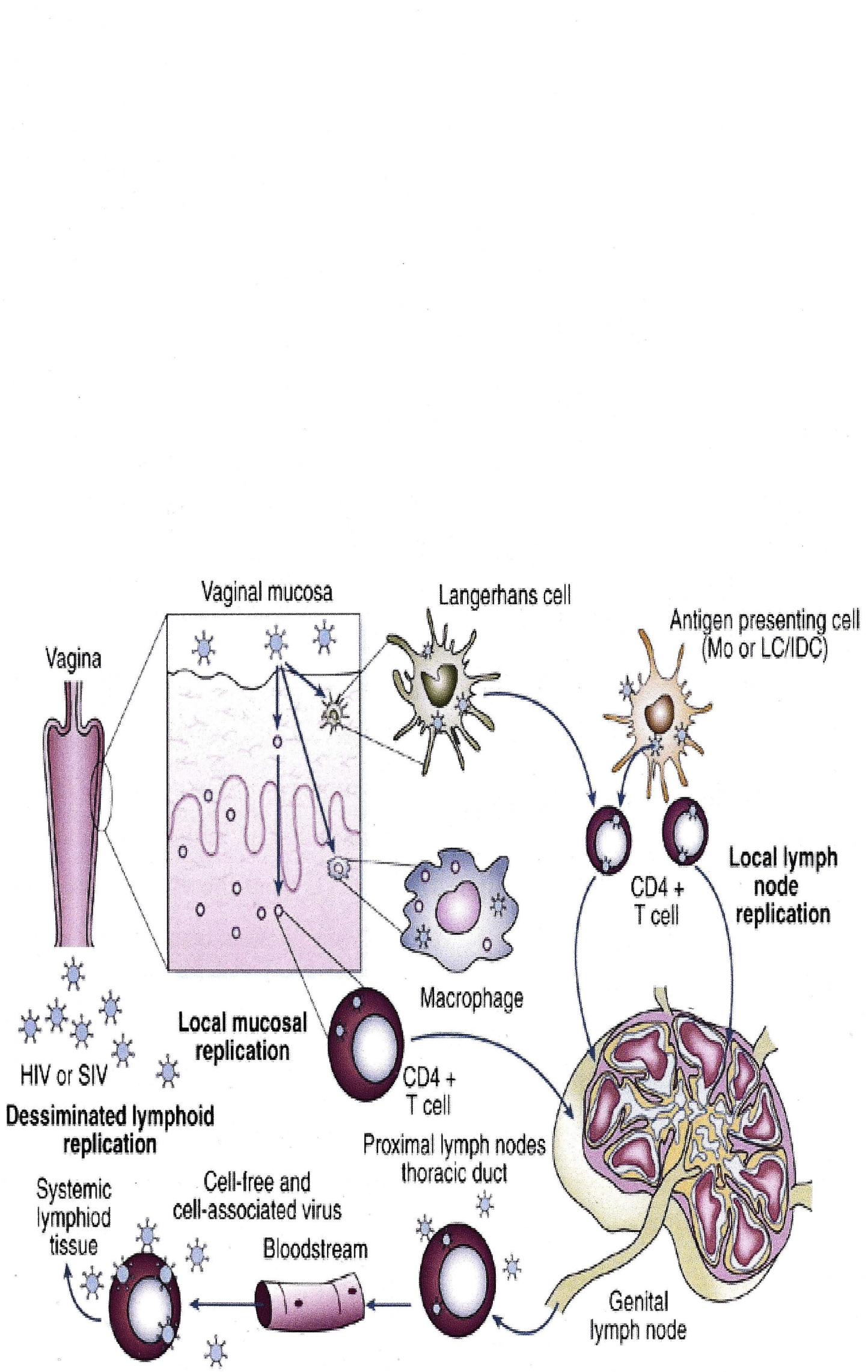



**T cells specific for vaccine antigen**

**Amplification of T cells specific to vaccine antigen**

**Fig 1.7: Schematic diagram depicting the pathogenesis of HIV**

*HIV infects $CD4^+$ T cells and LC of the genital mucosa. Inflammatory cytokines and chemokines induce HIV-infected LC, lamina propria DC and macrophages to migrate through the lymphatic vessels to the genital lymph nodes where the APC pass the HIV infection on to highly susceptible $CD4^+$ T cells. Once in the lymph node, the infection rapidly expands and disseminates through the lymphatic system to the blood and then to all lymphoid tissues. Figure reproduced from Miller et al.2007 [128].*


Vaginal mucosa
Langerhans cell
Antigen presenting cell
(Mo or LC/IDC)
Vagina
CD4 +
T cell
Local lymph
node
replication
Macrophage
Local mucosal
replication
HIV or SIV
CD4 +
T cell
Dessiminated lymphoid
replication
Proximal lymph nodes
thoracic duct
Systemic
lymphiod
tissue
Cell-free and
cell-associated virus
Bloodstream
Genital
lymph node

the mucosal compartment [136-138]. Furthermore, Ranasinghe *et al*. have demonstrated that compared to pure systemic (i.m/i.m) and pure mucosal (i.n/i.n) poxvirus prime-boost immunization, combined mucosal/systemic (i.n/i.m) immunization can generate robust T cell mediated immune response in both systemic and mucosal compartments [132] (Table 1.3). These studies indicate that route of delivery plays an important role in inducing systemic or mucosal immunity.

### 1.3.4 Avidity of T cells:

Many studies have shown that rather than the magnitude (number of IFN-γ producing HIV- specific T cells), the quality or avidity of T cells plays an important role in protective immunity [139-142]. Avidity can be explained as the strength of interaction between a T cell and the target antigen or the ability of the T cells to recognize MHC complexes on the target cells. In addition, high avidity CTLs recognize low concentration of MHC-I peptide complexes when compared to low avidity CTLs which are ineffective at recognizing low concentration of peptides on antigen presenting cells (Fig 1.8) [139, 143]. This suggests that high affinity CTL clear the viral infections more efficiently than the low CTL.

Studies in our laboratory, have shown that unlike systemic immunization (i.m/i.m), mucosal (i.n/i.n) and combined mucosal-systemic (i.n/i.m) immunizations can generate high avidity HIV-specific CTLs, where i.n/i.n > i.n/i.m > i.m/i.m (Ranasinghe et al., 2007) and the mucosally immunized animals were better protected against the mucosal influenza-HIV challenge (Ranasinghe et al., 2007). Furthermore recent, studies in our laboratory also indicate that compared to i.m. DNA-HIV/i.n. FPV-HIV poxvirus prime-boost immunization, i.n FPV-HIV/ i.m VV-HIV prime-boost vaccine can generate elevated high avidity $CD8^+$ T cells, with broader cytokine/chemokine profiles with excellent protective immunity [124, 144]. Belyakov *et al*. have also demonstrated that mucosal HIV-1 vaccine generates highly functional $CD8^+$ T cells in the gut mucosa compared to the systemic immunization, and these high avidity $CD8^+$ T cells are efficient at controlling HIV-1 viral replication in macaques [145]. These observations clearly indicate that the route of immunization not only influence the magnitude but also the quality of immune response.

The ability of T cells to secrete certain cytokines have shown to be crucial in influencing the avidity of $CD8^+$ T cells. For example, high avidity $CD8^+$ T cells are also

**Table 1.3: Route of vaccine delivery and magnitude of immune response**

| Vaccine delivery route | Mucosal response | Systemic response |
|---|---|---|
| Systemic immunization (i.m / i.m) | Low | High |
| Mucosal immunization (i.n / i.n) | High | Low |
| Combined mucosal and systemic immunization (i.n / i.m) | High | High |

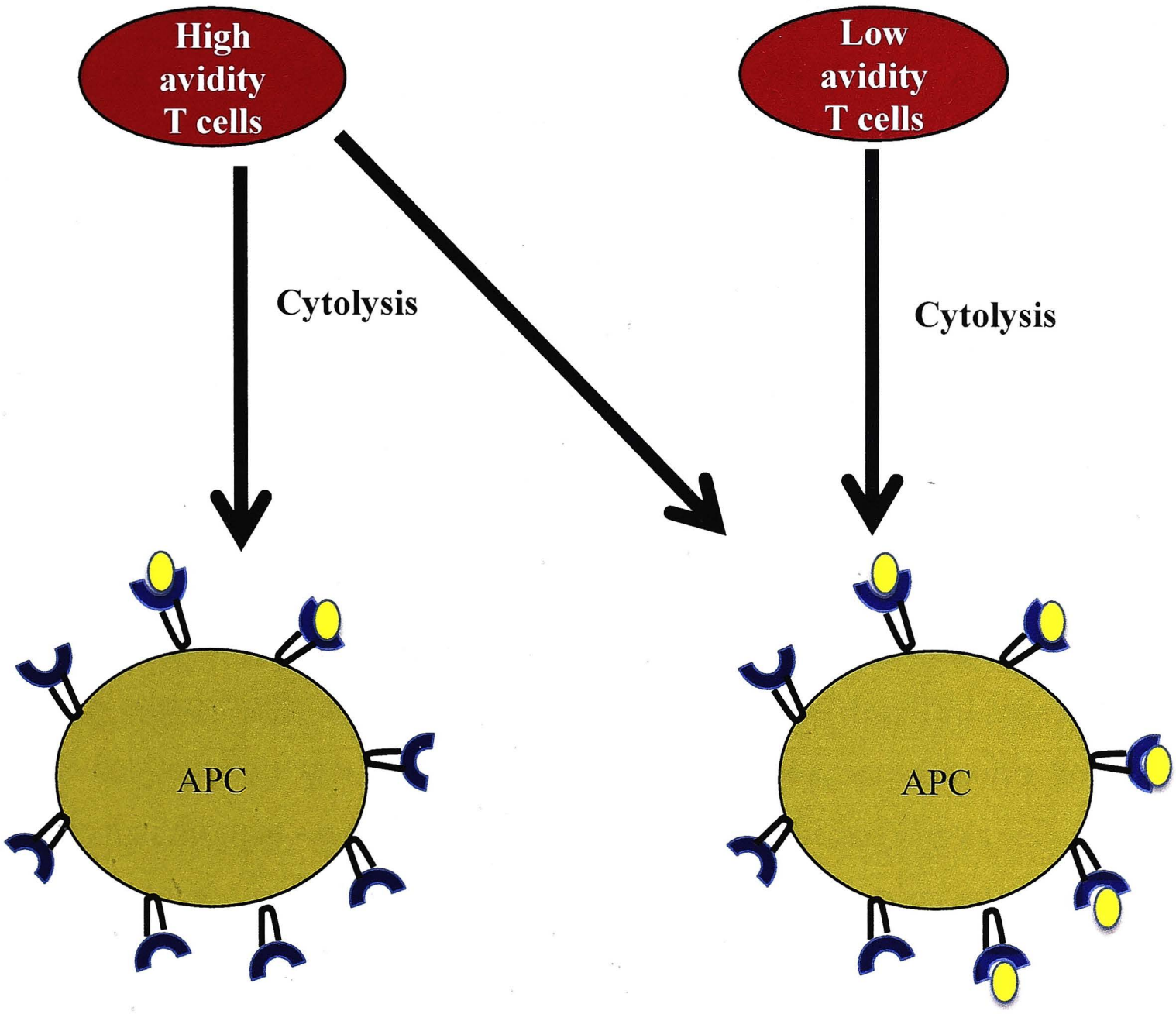
*BALB/c mice (n=4-5) were prime boosted with 1) i.n/i.n, 2) i.n/i.m, 3) i.m/i.m) following FPV-HIV.VV-HIV (expressing HIV gag pol genes). At 14 days post booster immunization, animals were sacrificed, spleens were harvested and single cell suspensions were prepared. Magnitude of systemic $CD8^+$T cell responses were measured by IFN-γ ELIspot [144].*

**Fig 1.8: Schematic diagram indicating the role of high and low avidity $CD8^+$ T cells**

*High avidity CTLs recognize low concentration of MHC I peptides on APC and are highly polyfinctional. In contrast, low avidity CTLs usually recognize much higher concentrations of MHC I peptides on APC and are poorly polyfunctional.*

- Highly Polyfunctional and express Th1 cytokines (IFN-γ, TNF-α, IL-2
- Induce rapid viral clearance and protective immunity

- Poorly Polyfunctional and express elevated Th2 cytokines
- Induce lower protection



thought to be multifunctional or polyfunctional and are able to produce range of Th1 (IFN-γ, IL-2 and TNF-α) cytokine upon peptide stimulation [96, 146, 147]. Similarly studies also have shown that low avidity $CD8^+$ T cells produce Th2 cytokines and become poorly functional (Kienzle et al., 2004, [144]

### 1.4.1 Modulation of HIV-specific $CD8^+$ T cell avidity by IL-4/IL-13 expression

In general, it is established that $CD8^+$T cells are unable to produce IL-4, however Kienzle *et al.* for the first time demonstrated that under certain *in-vitro* conditions $CD8^+$ T cells could produce IL-4 and influence the T cell avidity [148]. Similarly, IL-4 expressing $CD8^+$ T cells were also reported following dengue, natural mouse-pox and HIV infections [149] (Ranasinghe unpublished data). This atypical expression of IL-13/IL-4 is most likely an inherent property of these viruses to evade the immune system.

Studies in our laboratory have shown that following recombinant poxvirus prime-boost immunization, HIV-specific $CD8^+$ T cells can express Th2 cytokines IL-13 and IL-4 *in vivo* in a vaccine route dependent manner (i.n./i.n. < i.n./i.m. < i.m. I.m.) and the expression was inversely correlated with $CD8^+$ T cell avidity (Table 1.4) [144, 150]. This data indicated that mucosal vaccination induced high avidity HIV-specific $CD8^+$ T cells with lower IL-4/IL-13 with better protective immunity. Using IL-4, IL-13 KO mice Ranasinghe *et al.* have further substantiated that the total absence of IL-13 enhanced effector and memory $CD8^+$ T cell avidity (Ranasinghe & Ramshaw 2009a) and induce better protective immunity (Ranasinghe *et al.* Submitted-June 2012). These studies clearly indicated that designing HIV-1 vaccines that can induce high avidity of T cells offer great promise for the future.

### 1.4.2 IL-13 receptor and signaling

The IL-13 receptor system consists of IL-4 receptor alpha (IL-4Rα), and IL-13Rα1 also known as Type II receptor complex and IL-13Rα2 (decoy receptor) and also IL-13Rα2 soluble receptors. IL-13Rα1 itself has a low affinity binding to IL-13 which, when combined with IL-4Rα forms a highly functional IL-13 receptor that signals via STAT6 [151-154]. In contrast, IL-13Rα2 contains only a short cytoplasmic domain and has no known signaling function serving as an IL-13 decoy receptor (Fig 1.9)[152]. Typically, IL-13Rα2 exists as a membrane bound and a soluble form [155].

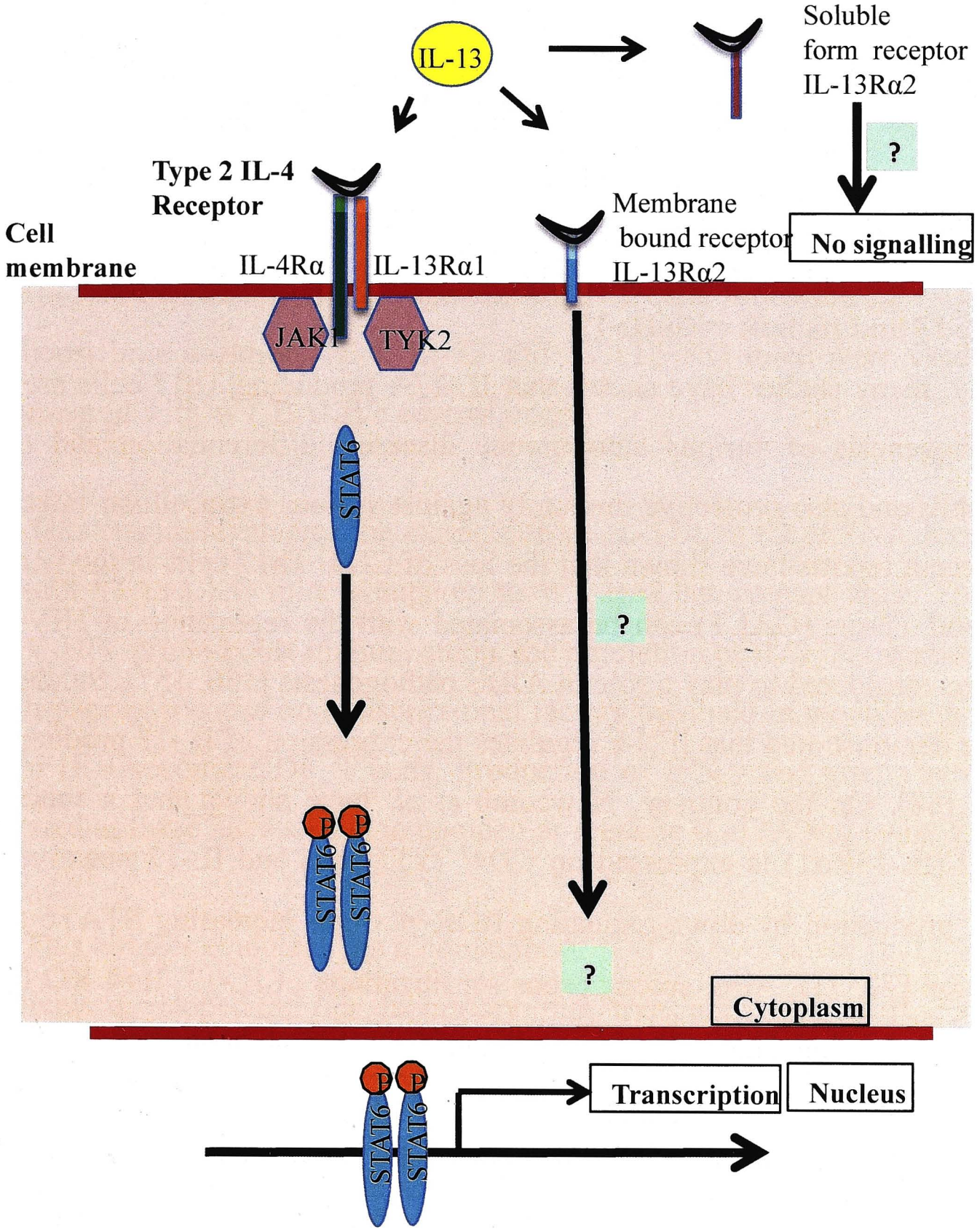
**Table 1.4: Magnitude and avidity of HIV specific $CD8^+$ T cells following route of vaccine delivery in BALB/c and IL-13-/- mice**

| Vaccine delivery route | Mice Strain | Magnitude of response (IFN-γ) | IL-4/IL-13 expression in HIV specific $CD8^+$ Tcells | CTL avi |
|---|---|---|---|---|
| i.n/i.n | BALB/c | Low | Low | High |
| i.n/i.m | BALB/c | High | Medium | Medium |
| i.m/i.m | BALB/c | High | High | Low |
| i.n/i.n | IL-13-/- | Low | Absent | High |
| i.n/i.m | IL-13-/- | High | Absent | High |
| i.m/i.m | IL-13-/- | High | Absent | High |

*BALB/c mice (n=4-5) were prime boosted with FPV-HIV/VV-HIV (expressing HIV gag pol genes). At 14 days post booster immunization, animals were sacrificed, spleens were harvested, and single cell suspensions were prepared. Magnitude of systemic $CD8^+$ T cell responses were measured by IFN-γ ELIspot, the expression of IL-4 and IL-13 by $K^dGag_{197-205}$ specific T cells by single cell multiplex RT-PCR and avidity of CTL by $K^dGag_{197-205}$ specific tetramer dissociation (Ranasinghe et al, 2009). Six weeks post booster immunization protection was evaluated following intranasal influenza virus encoding an HIV $K^dGag_{197-207}$ challenge, (+) indicates the level of protection evaluated by weight gain or loss. (Table adapted from Ranasinghe & Trivedi, 2010).*

**Fig 1.9: IL-13 receptors and signaling pathway**

*IL-13 signals through its complex receptor system including IL-4Rα, IL-13Rα1 and IL-13Rα2. IL-4Rα heterodimerize with IL-13Rα1 to form IL-13 receptor, activating STAT6 transcription. On the other hand, IL-13Rα2 serves as a decoy receptor and can be soluble or membrane bound form. Figure adapted from Tabata et al. 2007 [152].*


IL-13
Soluble
form receptor
IL-13Rα2
?
No signalling
Type 2 IL-4
Receptor
Cell
membrane
IL-4Rα
IL-13Rα1
Membrane
bound receptor
IL-13Rα2
JAK1
TYK2
STAT6
?
P
P
STAT6
STAT6
?
Cytoplasm
P
P
STAT6
STAT6
Transcription
Nucleus

To enhance the HIV-specific vaccine mediated $CD8^+$ T cell avidity, *Ranasinghe et al.* have recently designed a HIV vaccine, which co-expresses IL-13 soluble receptor IL-13Rα2Δ10 together with HIV *gag/pol* to temporarily block IL-13 expression at the vaccination site (in this vaccine the exon 10 of the receptor is deleted thus is referred to as Δ10). Using this vaccine in a prime-boost model, they have also shown that it can induce high avidity HIV-specific $CD8^+$ T cells with a broader cytokine/chemokine profiles with excellent protective immunity compared to the control vaccine (FPV-HIV/VV-HIV) (Ranasinghe *et al.*-submitted June 2012). Whether the novel vaccine can also modulate IL-17A expression in HIV-specific $CD8^+$ T cells will be investigated in this study.

### 1.4.3 IL-13 and its link with IL-17

Recently, many studies have shown that IL-17A producing Th17 cells are involved in the pathogenesis of various autoimmune diseases, differentiation and migration of neutrophils and also protective immunity against various extracellular infections (Table 1.1). Recent reports have shown that the loss of $CD4^+$ Th17 cells in the Gut Associated Lymphoid Tissue (GALT) can be associated with the replication of HIV-1 virus and therefore considered to play a role in AIDS pathogenesis [156, 157]. Studies by Cruz *et al.* have demonstrated that IFN-γ regulates the expression of IL-17 production in $CD4^+$ T cells [68]. On the contrary, Newcomb *et al.* have shown that a functional IL-13 receptor (IL-13Rα1) is expressed on $CD4^+$ Th17 cells and IL-13 negatively regulates IL-17A production by down-regulating ROR-γt while increasing STAT6 and GATA3 expression [70, 71]. Also, epicutaneous sensitizations of IL-13, IL-4 KO (Gene knock out) and IL-13 IL-4 DKO (Double gene knock out) mice have shown exaggerated IL-17A production in the lung [69]. In our laboratory, IL-4/IL-13 was shown to play an important role in HIV-specific $CD8^+$ T cell avidity. However, whether IL-13/IL-4/IFN-γ can modulate IL-17A expression in "HIV-specific $CD8^+$ T cells" in a prime-boost vaccination model is not known and this forms the basis of this project.

## 1.5 Hypothesis, aims and proposed research

**Hypothesis:** IL-17A expression in HIV specific $CD8^+$ T cells is mainly IL-4/IL-13 dependent and not IFN-γ and IL-17A plays an indirect role in modulating $CD8^+$ T cell avidity

**Aims**

**Aim 1**: To evaluate whether following HIV-1 prime-boost immunization the expression of IL-17A by HIV-specific $CD8^+$ T cells is regulated in an IL-4/IL-13, STAT6 dependent manner

**Aim 2:** To evaluate whether following novel IL-13 inhibitor vaccine; IL-17A plays a direct or indirect role in modulating HIV-specific $CD8^+$ T cell avidity and protective immunity.

**Chapter 3:** This chapter evaluates whether IL-4, IL-13 and STAT6 play a role in modulating IL-17A expression in HIV-specific $CD8^+$ T cells following FPV-HIV/VV-HIV prime-boost immunization. IL-4, IL-13 and STAT6 KO mice were used in this study and compared with WT BALB/c control mice.

**Chapter 4**: This chapter evaluates the expression of IL-17A at the mRNA level using IL-4, IL-13 and STAT6 KO mice compared to WT BALB/c control mice. Following FPV-HIV/VV-HIV prime-boost immunization, the regulation of IL-17A expression was assessed with particular focus on transcriptional factors involved in modulating IL-17A production in HIV-specific $CD8^+$ T cells. Production of IFN-γ and granzyme-B were also investigated as these have shown to dampen or regulate IL-17A and cytotoxicity.

**Chapter 5**: This chapter evaluates the modulation of IL-17A by a novel IL-13 inhibitor vaccine strategy developed in our laboratory that transiently inhibit IL-13 at the vaccination site. Immunizing WT BALB/c mice with novel IL-13 inhibitor vaccine, the expression of IL-17A in HIV-specific $CD8^+$ T cells was evaluated at different time intervals (acute, effector, memory and following challenge) to establish the involvement of IL-17A in protective immunity.

# CHAPTER 2: Materials and Methods

## 2.1 Materials

**Table 2.1**

| Cell lines | Description |
|---|---|
| 143B cell line (ATCC CRL-8303) | Human osteosarcoma cell line |
| CV-1 cell line (ATCC CCC-70) | African green monkey cell line derived from kidney cells |

**Table 2.2.**

| Buffers/solutions | Components | Source |
|---|---|---|
| Red cell lysis buffer | 0.16 M Ammonium chloride, 0.17 M Tris HCl | Prepared in the lab |
| FACS buffer | PBS 950 ml+<br>FCS 50 ml | Prepared in the lab |
| 1x Intracellular fixation buffer (IC-FIX) | 4% Paraformaldehyde + PBS | Biolegend |
| 1x Permeabilization buffer | 10x permeabilization buffer (100 ml) + $ddH_2O$ (900 ml) | eBiosciences |
| FACS sample resuspension buffer | PBS+ 0.5% Paraformaldehyde | Prepared in the lab |
| Wash buffer | PBS + 0.05%Tween 20 | Prepared in the lab |
| 1x Brefeldin A solution (1000x) (BFA) | 1:1000 dilution in Complete RPMI media | eBiosciences |
| Tryphan Blue (0.4%) | 1:5 dilution in PBS | Invitrogen |
| Complete RPMI medium | RPMI 1640<br>FCS 5% +<br>50 mM HEPES<br>6.5 nM sodium pyruvate<br>50 µg/ml Streptomycin<br>30 µg/ml Penicillin<br>50µM 2-mercaptoethanol | GIBCO<br>Invitrogen<br>Invitrogen<br>Invitrogen<br>Media wash-up<br>Media wash-up<br>Sigma |
| Minimal Essential Medium (MEM) | MEM+<br>FCS 5%<br>50 mM HEPES<br>50 µg/ml Streptomycin<br>30 µg/ml Penicillin<br>50 µM 2 mercaptoethanol | GIBCO<br>Invitrogen<br>Invitrogen<br>Media wash-up<br>Media wash-up<br>Sigma |
| Lung digestion buffer | Complete medium+<br>2 mg/ml Collagenase<br>2.4 mg/ml Dispase<br>5 U/ml DNAse | <br>Sigma<br>GIBCO<br>Calbiochem |

## 2.3 Antibodies

| Name | Working dilution | Source | Catalogue number | Experimental use |
|---|---|---|---|---|
| Anti CD8 APC | 1:300 | BD Pharmingen | 553035 | Binds to CD8 during surface staining in intracellular cytokine staining |
| Anti CD8 FITC | 1:200 | BD Pharmingen | 553031 | Binds to CD8, used in tetramer staining |
| Anti CD8 PE | 1:1000 | BD Pharmingen | 553033 | Binds to CD8 during surface staining in intracellular cytokine staining |
| Tetramer APC | 0.3 µl/well | BRF (JCSMR) | 09/07 | Used in tetramer staining |
| Anti-IFNγ FITC | 1:200 | eBiosciences | 11731182 | Binds to IFN-γ during ICS |
| Anti IL-17 APC | 1:100 | Biolegend | 506916 | Binds to IL-17 during ICS |
| Anti ROR-γt PE | 1:100 | eBiosciences/ Jomar bioscience | 12-6988-82 | Binds to ROR-γt during ICS |
| CD8 cocktail | 1:20 | Stem cell | 19753 | Binds to cell surface Ag CD4, CD 11b,CD-19, CD-45R, CD-49b, TER119 |
| Biotin cocktail | 1:10 | Stem cell | 19753 | Binds to bispecific tetrameric antibody complexes |
| Magnetic nanoparticle | 1:20 | Stem cell | 19753 | Binds to the magnetic labeled biotin cocktail |
| Mouse IL-17 Capture antibody | 1:250 | eBiosciences | 167175-85 | Binds to IL-17 during ELIspot |
| Mouse IL-17 Detection antibody | 1:250 | eBiosciences | 133-7177-68 | Detects IL-17 bound to capture antibody |

*APC=Allophycocyanin,
FITC=Fluoresceine-isothiocyanate,
PE=Phycoerythrer

## 2.4 Reagents used in RNA extraction and cDNA preparation

| **Buffers/Reagents** | **Components (Final Concentration)** | **Source** |
|---|---|---|
| TRI Reagent | 1 ml for up to $1x10^7$ cells/ml | Sigma |
| Chloroform | 200 µl | UNIVAR |
| 5x First strand buffer | 2 µl | Invitrogen |
| DNase | 1U/µl | Roche |
| Superscript III | 200 U/µl | Invitrogen |
| dNTP | 10 mM | Sigma |
| Oligo dT | 100 µM | Sigma |
| 2x Hotstar *Taq* Master mix | Final concentration used 1x | QIAGEN |

## 2.2 Methods:

### 2.2.1. Recombinant Poxvirus Vaccines

Recombinant viruses fowl pox (FPV) and vaccinia virus (VV) were used as vaccine vectors. The parent recombinant FPV and VV viruses expressing HIV gag/pol genes were obtained from Dr. David Boyle and rFPV vaccines were grown in our laboratory by Dr. Ronald Jackson [158, 159]. Also, the vaccines FPV-HIV and VV-HIV co-expressing IL-13Rα2 were constructed by Dr. Ronald Jackson using established recombinant pox viral cloning techniques regularly used in the laboratory (Fig 2.1 and table 2.5).

### 2.2.2 Preparation of recombinant vaccinia virus vaccine (rVV):

The roller bottles (Corning) were seeded with CV-1 ($1x10^8$) cells in MEM media and incubated at 37°C. Once the cells reached confluency, they were infected with vaccinia virus at 0.05 Multiplicity Of Infection [53] in 50 ml of media and incubated for 45 mins. Following incubation, 200 ml of MEM media was added and the cells were incubated for further 48-72 hrs at 37°C. To harvest the virus, cells were scraped off from the bottles, placed in 50 ml tubes, and centrifuged at 3000 rpm for 10-15 mins at 4°C. The cell supernatants were discarded and the pellets were resuspended in 5 ml PBS per roller bottle. Cells were sonicated 3x at 9 V (volts) for 20 secs and 1 ml aliquots were prepared and stored at -70°C.

### 2.2.3 Titration of recombinant vaccinia virus vaccine (rVV):

To determine the virus titer, rVV were titrated using 143B cells. Firstly, 143B cells were seeded onto 6 well plates and incubated overnight or until cells reached 80% confluency. The rVV virus was serially diluted (10 fold) and 100 µl of each dilution was added to 143B cells for 1 hr. Following incubation, 2 ml of MEM media was added on to each well and the cells were further incubated for 48 hrs. The media was removed and the cells were stained with 0.1% crystal violet in 20% ethanol to visualize the plaques. The plaques were then counted and the titer of the virus stock was determined.

**Fig 2.1: Schematic diagram of recombinant fowl-pox and vaccinia virus vaccines**

*Fig a) represents control vaccine encoding HIV gag/pol genes and Fig b) represents IL-13 Inhibitor vaccine co-expressing HIV gag/pol genes and soluble IL-13Rα2.*

**Fig a) Control vaccine**

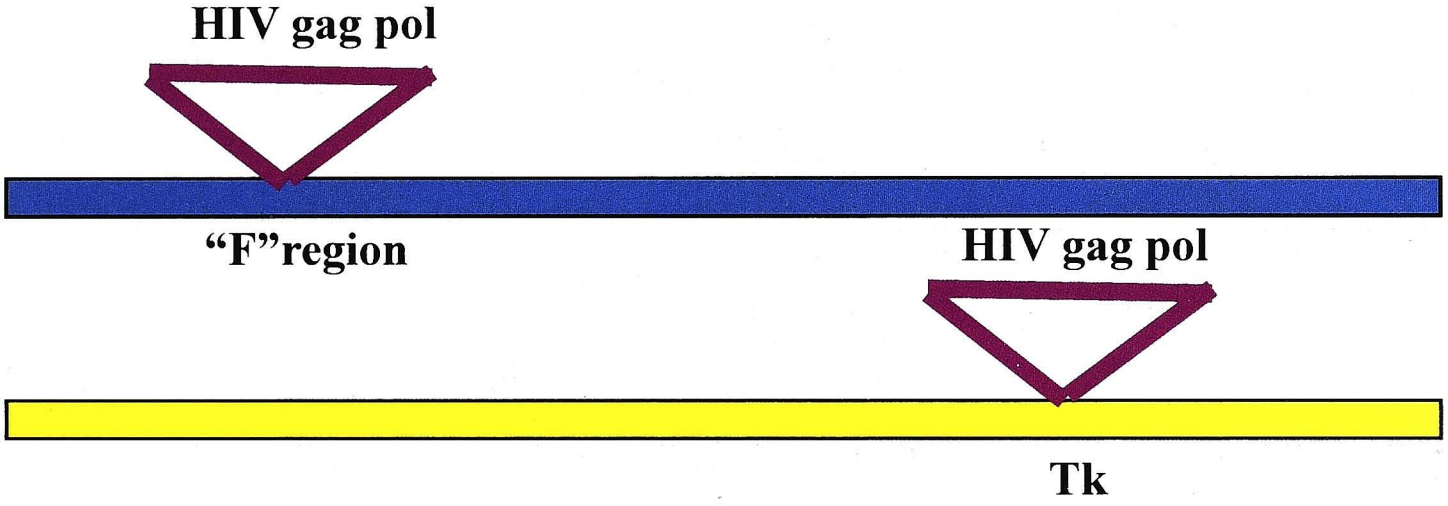



**Fig b) Novel IL-13 inhibitor vaccine**

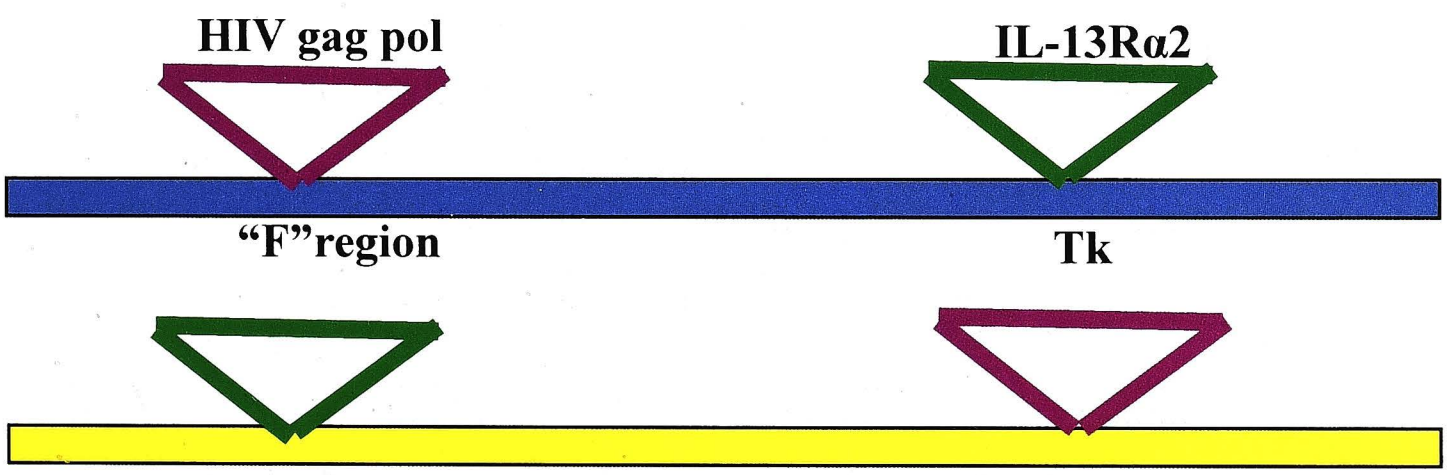


*Table 2.5: Lists of recombinant viruses and their gene insertion sites (Coupar et al, 2006)*

| **Recombinant viruses** | **Insertion sites** | |
|---|---|---|
| | *F Region* | *Tk-ORFX or Tk* |
| *FPV-HIV (086)* | *gag / pol* | - |
| *VV-HIV (336)* | - | *gag / pol* |
| *FPV –HIV IL-13Rα2* | *gag / pol* | *IL-13Rα2* |
| *VV-HIV IL-13Rα2* | *IL-13Rα2* | *gag / pol* |

*Tk=Thymidine Kinase, ORFX=Uncharacterized gene*

### 2.2.4 Immunization of mice

Pathogen free, 6-8 weeks old female wild type BALB/c (H-$2^d$), IL-13-/-, IL-4 -/- and STAT6-/- knock out mice (KO) on BALB/c background were obtained from the Australian National University (ANU) animal breeding facility. These animals were used and maintained in accordance with the ANU animal experimentation ethics guidelines (Table 2.6). For challenge studies, spleen and lung samples were obtained from Dr. Charani Ranasinghe. The weights of unimmunized and immunized animals were similar to that observed in Ranasinghe *et al.*2011 **[124]**.

Mice (n=5-10) were prime-boost immunized with either 1 x $10^7$ pfu FPV-HIV or FPV-HIV IL-13Rα2 followed by 1 x $10^7$ pfu VV-HIV or VV-HIV IL-13Rα2, 2 weeks apart using i.n/i.m (combined mucosal systemic immunization route).

During intranasal immunization (i.n), mice were anaesthetized under methoxyflourane and ~10 µl of virus was delivered to each nostril (total not exceeding 25 µl). For intramuscular immunization (i.m), mice were first injected intra-peritoneally (I.P.) with 200 µl of avertin (Fluka) as a general anesthetic and mice were then immunized with 50 µl per leg (quadriceps muscle) of respective vaccines.

Following prime-boost immunizations, mice were sacrificed at different time intervals (3 days, 14 days, 8 weeks, challenge) and systemic and mucosal T cell response were measured in spleen and lung samples respectively (Fig 2.2).

### 2.2.5 Peptides used:

In intracellular staining, ELIspot and RT-PCR assays, cells were stimulated with immunodominant H-2K$^d$ binding 9 mer HIV Gag peptide $^{197}$AMQMLKETI$^{205}$, synthesized at the Biomolecular research facility (BRF), JCSMR.

### 2.2.6 Preparation of splenic lymphocytes:

To evaluate systemic T cell responses in spleens at acute, effector and memory stages of immune response, mice were sacrificed at 3 days, 14 days and 8 weeks following prime boost immunization respectively (Fig 2.2). Single cell suspensions of spleen samples were prepared by passing spleen through Falcon $^{TM}$ Nylon cell strainer into RPMI medium (Table 2.2) using a syringe plunger. Cells were then washed and resuspended in red cell lysis buffer for 5-7 mins at room temperature. Cells were diluted with 20 ml

**Fig 2.2: Vaccination-time line:**

*Schematic diagram of the vaccines used and the time schedule.*

2 weeks | 3 days | 14 days | 8 weeks | 10 days

**i.n**
1x $10^7$ PFU
rFPV-HIV (or)
r FPV-HIV-IL-13Rα2

**i.m**
1x $10^7$ PFU
rVV-HIV (or)
rVV-HIV-IL-13Rα2

| Acute T cell imunity | effector T cell imunity | Memory- T cell immunity | Influenza-Challenge |
|---|---|---|---|

**Table 2.6: List of mice and vaccines that were used in this study**

| Mice | Vaccines | |
|---|---|---|
| | ***Prime*** | ***Boost*** |
| ***BALB/c*** | ***FPV-HIV*** | ***VV-HIV*** |
| ***BALB/c*** | ***FPV-HIV IL-13Rα2*** | ***VV-HIV IL-13Rα2*** |
| ***IL-13 KO*** | ***FPV-HIV*** | ***VV-HIV*** |
| ***IL-4 KO*** | ***FPV-HIV*** | ***VV-HIV*** |
| ***STAT-6 KO*** | ***FPV-HIV*** | ***VV-HIV*** |

*HIV = gag pol genes*

*FPV = Fowl pox virus*

*VV = Vaccinia virus*

*IL-13Rα2 = IL-13 Soluble receptor*

of RPMI media and were centrifuged at 1200 rpm for 5 mins at 4°C. Cells were then washed twice in RPMI medium. Cell pellets were resuspended in a final volume of 5 ml complete RPMI medium, and were counted using Neubauer haemocytometer.

### 2.2.7 Preparation of lung samples:

Lung samples were cut into small pieces and digested with 1-2 ml of lung digestion buffer as described in table 2.2. The samples were then incubated at 37°C in a water bath for 1 hr with gentle vortexing every 15 mins. The digested cell suspensions were passed through a Falcon™ Nylon cell strainer into RPMI medium. Cells were then resuspended in red cell lysis buffer for 5-7 mins at room temperature, samples were diluted with RPMI medium and were centrifuged at 1200 rpm for 5 mins at 4°C. The samples were then filtered through sterile gauze to remove connective tissue and debris. Following filtration, the samples were washed twice in complete RPMI and the cell pellets were resuspended in 1-2 ml of complete RPMI and the cells were counted.

### 2.2.8 Tetramer staining

Tetramer staining was performed to evaluate the number of HIV-specific $CD8^+$ T cells. 2 x $10^6$ cells were added into 96 well U–bottom plates and were washed with 100 µl FACS buffer. Diluted fluorescein isothiocynate (FITC) conjugated anti CD8 antibody (1:200 final dilution) and 0.3 µl of APC labeled Gag $^{197}$AMQMLKETI$^{205}$ H-2K$^d$ restricted tetramer (synthesized at the BRF-JCSMR) was added at a final volume of 40 µl, and cells were incubated at room temperature in the dark for 45 mins. Following incubation, cells were washed twice and resuspended in 100 µl of FACS buffer. Tetramer specific $CD8^+$ T cells were sorted using FACS LSR ARIA-II.

### 2.2.9 Intracellular Cytokine Staining of IFN-γ and IL-17:

For intracellular cytokine staining, 4 x $10^6$ cells/well were aliquoted into a 96 well U bottom plates and stimulated with immunodominant H-2K$^d$ binding $^{197}$AMQMLKETI$^{205}$ 9 mer Gag peptide (5 µg/ml of final concentration) for 16 hrs at 37 °C with 5% $CO_2$. Brefeldin-A (1:1000 (1000x stock) was added and the samples were further incubated for 4-5 hrs. Cells were washed with FACS buffer and firstly stained with anti-CD8 PE (1:1000 dilution) (B.D Pharmingen) diluted in FACS buffer and incubated for 30 mins at 4°C. Unbound antibodies were removed by washing with FACS buffer and cells were

then fixed with 100 µl of 1x IC -FIX for 10 mins on ice. Cells were then washed and permeabilized by resuspending in 100 µl IC-PERM for 10 mins at room temperature. Cells were spun down and conjugated anti-cytokine antibodies were diluted in IC-PERM (IFN-γ FITC 1:200 and IL-17 APC 1:100). Cells were then resuspended in 25 µl diluted antibodies, and incubated on ice for 30 mins. Then, the cells were washed twice with FACS buffer, resuspended in 100 µl of 0.5% paraformaldehyde and were transferred to cluster tubes (Costar). Samples were run on a four color FACS Calibur Flow cytometer (Becton and Dickinson) until 500,000 events were acquired. Data were analyzed using Cell Quest software using the gating strategy as described in Chapter 3. The percentages of the HIV-specific CD8 cytokine producing T cells were calculated by subtracting the stimulated values from the unstimulated background.

### 2.2.10 Negative selection of $CD8^+$ T cell

To enrich $CD8^+$ T cell, single cell suspensions of spleen cells were prepared as described previously (section 2.2.6). Negative selection was performed according to the manufacturers instructions (stem cell). Cell densities were adjusted to 1 x $10^8$ cells/ml in PBS supplemented with 1% FCS. The cells were then transferred to 5 ml Falcon™ polysterene round bottom tubes. To each tube, CD8 T cell enrichment cocktail (1:20 dilution) was added and incubated at 4°C for 15 mins. Following incubation, Biotin selection cocktail (1:10 dilution) was added, mixed and incubated at 4°C for 15 mins. Magnetic nanoparticles were mixed thoroughly to ensure uniform suspension and was added (1:20 dilution) to the samples and further incubated at 4°C for 15 mins. The cell suspensions were made up to 2.5 ml with FACS buffer. The samples were mixed and placed in the Easysep Magnet (Stem cell) for 5 mins. Following incubation, cells were collected in a fresh 5 ml polysterene tubes while the magnetically labeled unwanted cells remained bound to the original tube by the magnetic field. Isolated $CD8^+$ T cells were pooled, washed and resuspended in complete RPMI medium. Both enriched and non-enriched samples were surface stained and were analyzed using FACS calibur for purity (Fig 2.3).

### 2.2.11 IL-17A (Enzyme Linked ImmunoSPOT) ELIspot

Spleen and lung cells were assayed for IL-17A expression by HIV Gag-specific $CD8^+$ T cells as described by manufacturer (e-Biosciences). Briefly, Multiscreen PVDF filter plates (Millipore, USA) were coated overnight at 4°C with 4 μg/ml concentration (50 μl/well) of anti-mouse IL-17 capture antibody. The plates were washed 3x with PBS with 0.05% Tween (PBST) and 2x with PBS, and then 200 μl of complete RPMI medium was added to each well and the plates were incubated at 37°C for 1 hr with 5% $CO_2$. To measure HIV-specific responses, enriched spleen cells and non-enriched lung cells at a density of 4 x $10^6$ cells/ml in 100 μl were added to the plates in duplicates. Cells were then stimulated with (5 μg/ml) immunodominant H-$2K^d$ binding $^{197}$AMQMLKETI$^{205}$ 9 mer Gag or with (5 μg/ml) Con-A as a positive control. Unstimulated cells were included as negative control. The plates were then incubated at 37°C with 5% $CO_2$ for 20-22 hrs. The cells were then removed from the plate and to lyse any remaining cells, 200 μl of ice-cold $ddH_2O$ was added to each well for 10 mins then washed 3x with PBST and 2x with PBS. To each well, 2 μg/ml of biotinylated anti-mouse IL-17 antibody in assay diluent (1x,eBiosciences) was added and the plates were incubated at room temperature for 2 hrs. The plates were washed as before and streptavidin alkaline phosphatase (1:1000) (GE Healthcare) was added and incubated for 90 mins at room temperature. The plates were again washed and 50 μl/well of BCIP/NBT alkaline phosphatase substrate (Moss Inc, USA) was added and incubated for 20-30 mins to develop the Spot forming units (SFU). The plates were finally washed under tap water and were allowed to dry. The SFU were counted using ELIspot Bioreader-4000 PRO-X (BioSys, Germany).

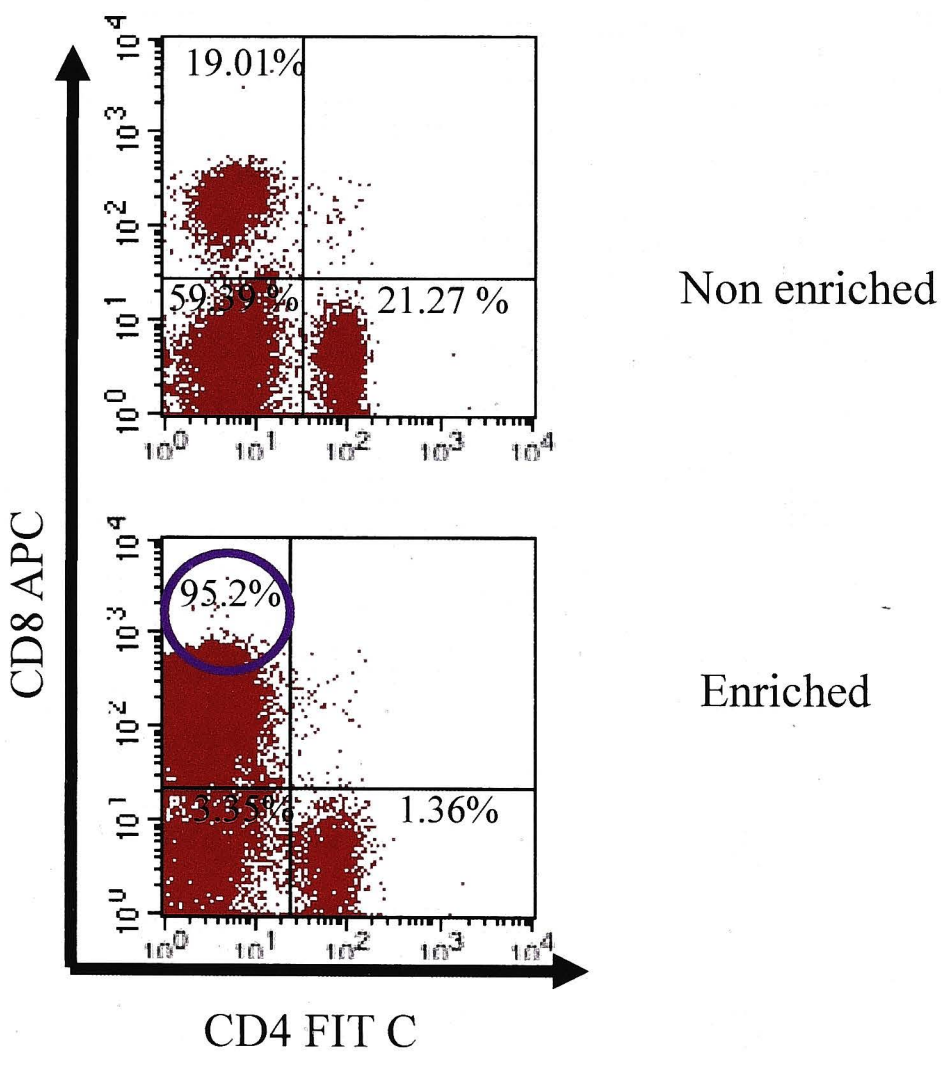
## 2.3. Molecular techniques

### 2.3.1 RNA isolation

Spleen cells were counted and $CD8^+$ T cells were enriched as described in 2.2.10. Samples were stimulated with 5 μg/ml immunodominant H-$2K^d$ binding $^{197}$AMQMLKETI$^{205}$, 9 mer Gag peptide for 4 hrs and 16 hrs respectively. Following stimulation, cells were gently aliquoted into RNase free eppendorf tubes and were centrifuged for 5 mins at 1500 rpm. The supernatants were removed and cell pellets were snap frozen on dry ice and stored at -70°C until use or 1 ml of TRI reagent was added to the cell pellet and were mixed by pipetting several times to break the cells

**Fig 2.3: CD8$^+$ T cell enrichment**

*Spleen cells were harvested from BALB/c mice and single cells suspensions of spleen cells were prepared. CD8$^+$ T cells were enriched as explained in section 2.2.10. 2 x $10^6$ cells of both enriched and non-enriched samples were surface stained with anti CD8 APC and anti CD4 FIT C and the samples were run in FACS calibur and analyzed using cell quest software.*




Non enriched

Enriched

(Table 2.4). Then, the samples were incubated on ice for 15 mins and 200 µl of chloroform was added into each tube, mixed and further incubated on ice for 15 mins. The samples were then centrifuged at 12000 rpm for 15-30 mins at 4°C (Microfuge centrifuge, Beckman Coulter), after which the top aqueous phase was removed carefully from each sample and transferred into fresh tubes and equal volume of isopropanol was added, mixed and the samples were snap frozen on dry ice for 30 mins. The samples were centrifuged at 8000 rpm for 30 mins at 4°C to pellet the RNA. Finally, the supernatants were removed and pellets were washed in 500 µl of 75% ethanol. The pellets were air-dried and resuspended in 20 µl of RNase free dd$H_2O$, and stored at -70°C.

### 2.3.2 DNAse treatment and cDNA synthesis

For the reverse transcription of RNA into cDNA, the frozen RNA samples were quantitated using Nanodrop$^R$ ND 1000 Spectrophotometer software. Then the RNA samples were treated with DNAse-I (Roche) to degrade the contaminating DNA. For each 1-3 µg of RNA, 1 µl of DNase (1 U/µl) and 2 µl of 5x first strand buffer was added and mixed gently. Samples were first incubated at 37°C for 30 mins, spun down again and further incubated at 75°C for 5 mins to degrade DNase I enzyme.

The samples were quantitated as before using Nanodrop to obtain the real concentration of RNA. For each reverse transcription reaction, 1-3 µg of RNA and dd$H_2O$ to a total of 10 µl was aliquoted into eppendorf tubes and 1 µl of oligo dT primer (100 µM), 1 µl dNTP (containing 10 mM each of dATP, dGTP, dCTP, and dTTP) and 3 µl RNase free dd$H_2O$ was added. Samples were then incubated at 65°C for 5 mins to remove the secondary RNA structure and then the samples were spun down, cooled on ice for 2 mins and to each cDNA synthesis reaction, 2 µl of 5x first strand buffer, 2 µl of DTT (0.1M), and 1 µl superscript III reverse transcription enzyme was added. Samples were mixed and then incubated first at 50°C for 50 mins, and then 70°C for 15 mins to inactivate the superscript enzyme (Table 2.4).

### 2.3.3 Pre-amplification of cDNA

As the expressions of the cytokines of interests (IL-6, IL-17, IL-23a) and transcription factors (T-bet, TGF-β, GATA-3 and ROR-γt) were found to be lower, where appropriate the samples were pre-amplified with gene specific primers using Hot-star

Taq Master mix (Table 2.7) [144, 160]. Briefly, 5 μl of Hot-star Taq Master mix, 3 pmol of each of forward and reverse primer and 1 μl of the synthesized cDNA was mixed and the samples were amplified at 95°C for 2 mins followed by 18 cycles of 95°C for 15 sec and 60°C for 4 mins. The pre-amplified cDNA was diluted 1:5 and 2 μl of the diluted sample was used in real time PCR.

### 2.3.4 Real time PCR

Real-time PCR was performed, using Applied Bio-systems SYBR Green PCR master mix (containing SYBR green 1 Dye, AmpliTaq Gold ® DNA polymerase, dNTPs with dUTP, passive reference ROX$^{TM}$ and buffer). Reactions were performed in eppendorf twin-tec 380 well PCR plates in 10 μl reaction volume containing 5 μl master mix, 3 pmol of each forward and reverse primer and 2 μl of preamplified cDNA. All primers were designed to have Tm of 60°C and the reactions were run at 1x 50°C for 2 mins, 1 x 95°C for 10 mins, followed by 40 cycles of 95°C for 15 secs, 60°C for 1 minute on an ABI PRISM Q 7900 sequence detection system (Applied Bio systems) and analyzed using SDS 1.9.1 software. Fold increase in mRNA were calculated after normalizing against house keeping gene; L32 expression, using the formula described in **[144]**.

Fold change = $2^{(-_{\Delta\Delta}Ct)}$, where $\Delta\Delta Ct = (Ct\ target\text{-}Ct\ reference)_{stimulated} -$

$(Ct\ target\text{-}Ct\ reference)_{unstimulated}$

arbitrary copy numbers were calculated using the formula as described in [161]

arbitrary copy number = $(10^5/2^{(Ct-17)}) \times 10^4$, where 1 Ct value = $10^5$ mRNA copies.

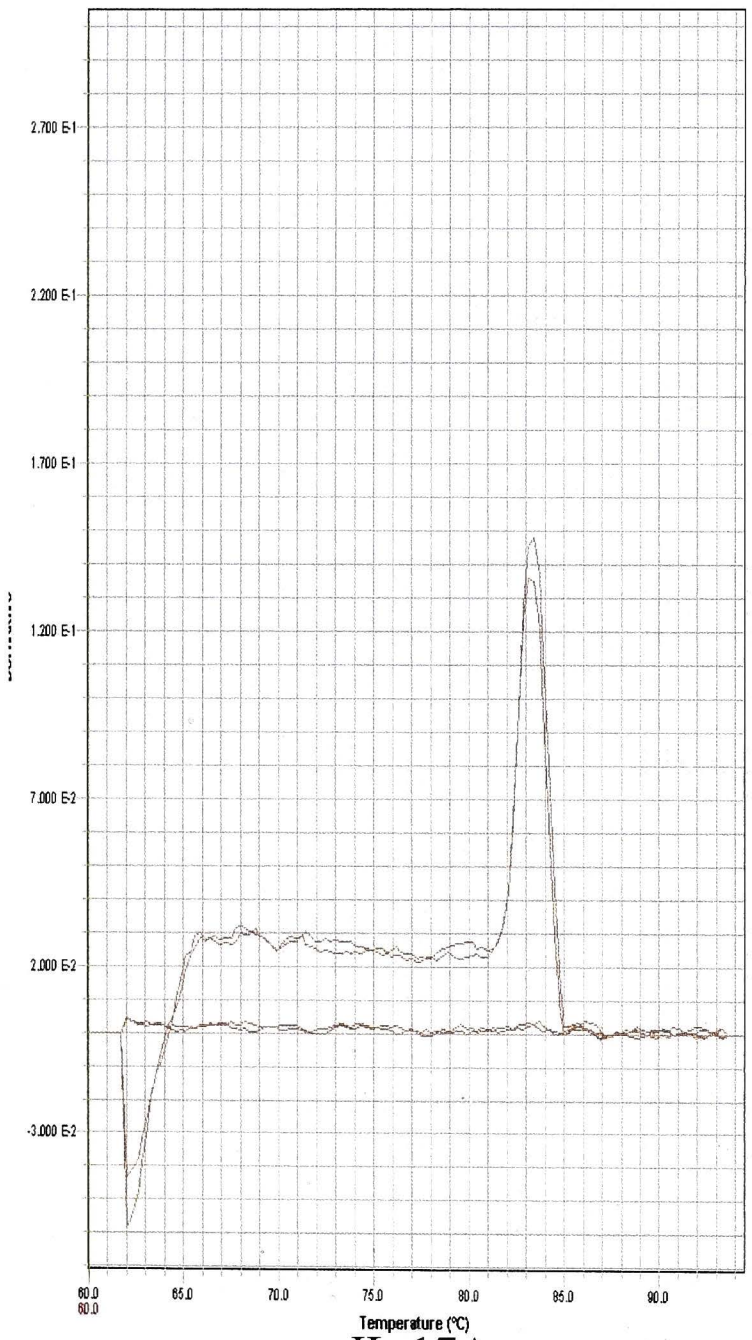
The specificity of the real time product was analyzed by performing dissociation curves at 95°C for 15 seconds, 60°C for 20 seconds followed 95°C for 20 mins during which the dissociation data was collected. The specificity of the product amplification was indirectly assessed by the analysis of dissociation curves (Fig 2.4).

### 2.3.5 Synthesis of cDNA from tetramer specific CD8$^+$ T cells:

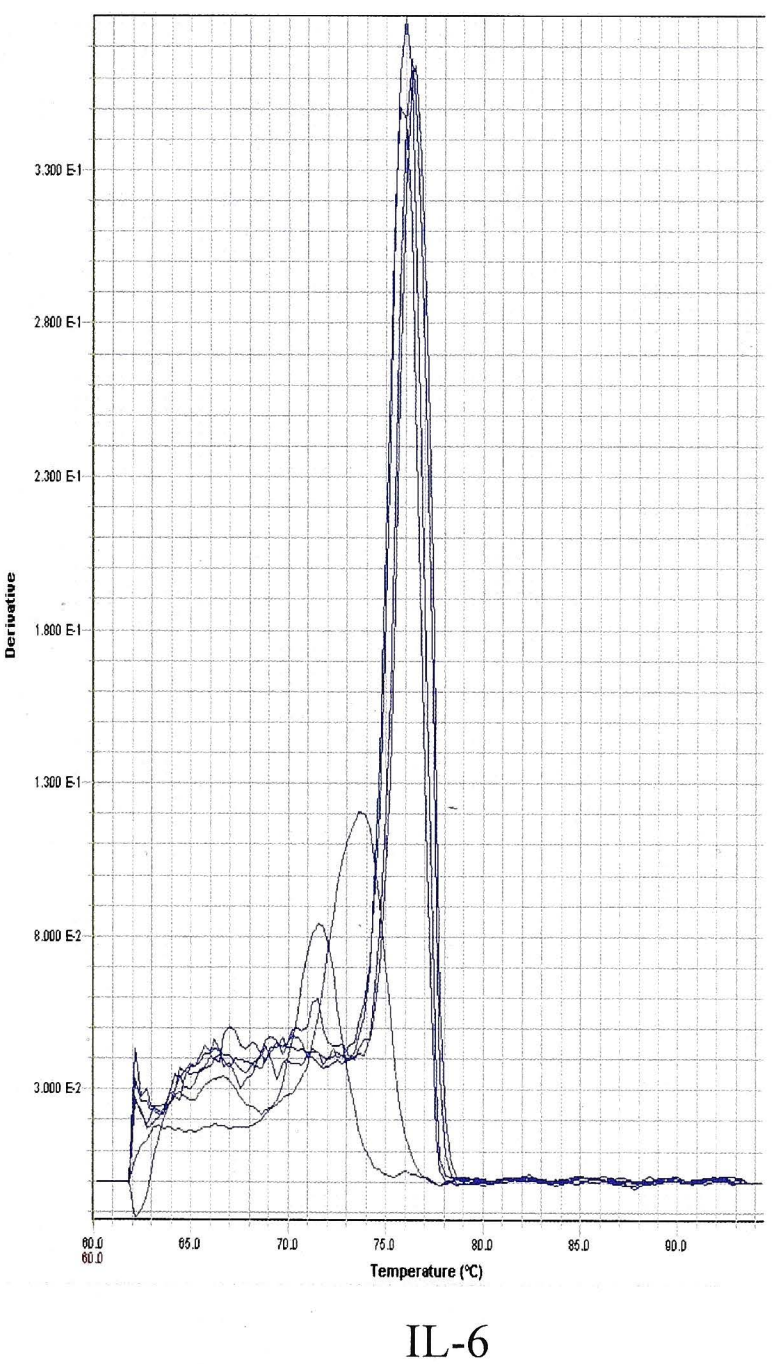
To examine the mRNA expression profiles IFN-γ, IL-17, granzyme-B, by HIV-specific CD8$^+$ T cell using real time PCR was performed on $K^d$ Gag $_{197\text{-}205}$ specific CD8$^+$ T cells. Firstly, spleen cells from each group were pooled and $K^d Gag_{197\text{-}205}$ specific tetramer staining was performed as described in 2.2.8. 96 well plates were prepared as follows,

**Fig 2.4: Dissociation curves and standard curves for qPCR with specific primers**

*The figures illustrate the dissociation curves of specific primers such as IL-6, IL-17A, IL-23a, T-bet, GATA3, ROR-γt. The peak in the melting curve represent melting temperature of each primer and y-axis indicates derivative or reaction data, which is the rate of change in fluorescence.*


2.700 E-1
2.200 E-1
1.700 E-1
1.200 E-1
7.000 E-2
2.000 E-2
-3.000 E-2
60.0
60.0
65.0
70.0
75.0
80.0
85.0
90.0
Temperature (°C)

IL-17A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2.800 E-1
2.300 E-1
1.800 E-1
1.300 E-1
8.000 E-2
3.000 E-2
Derivative
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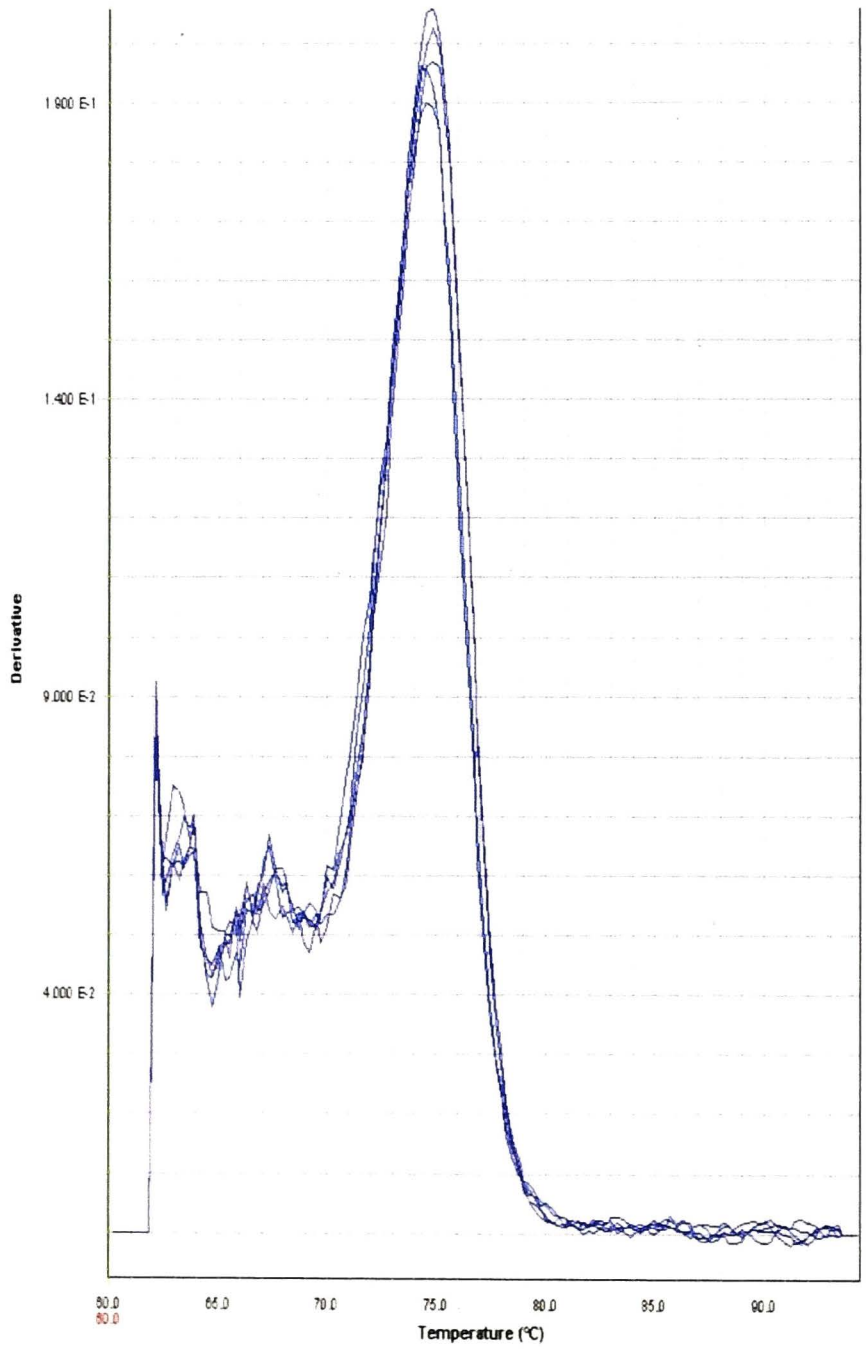
60.0
65.0
70.0
75.0
80.0
85.0
90.0
Temperature (°C)

IL-6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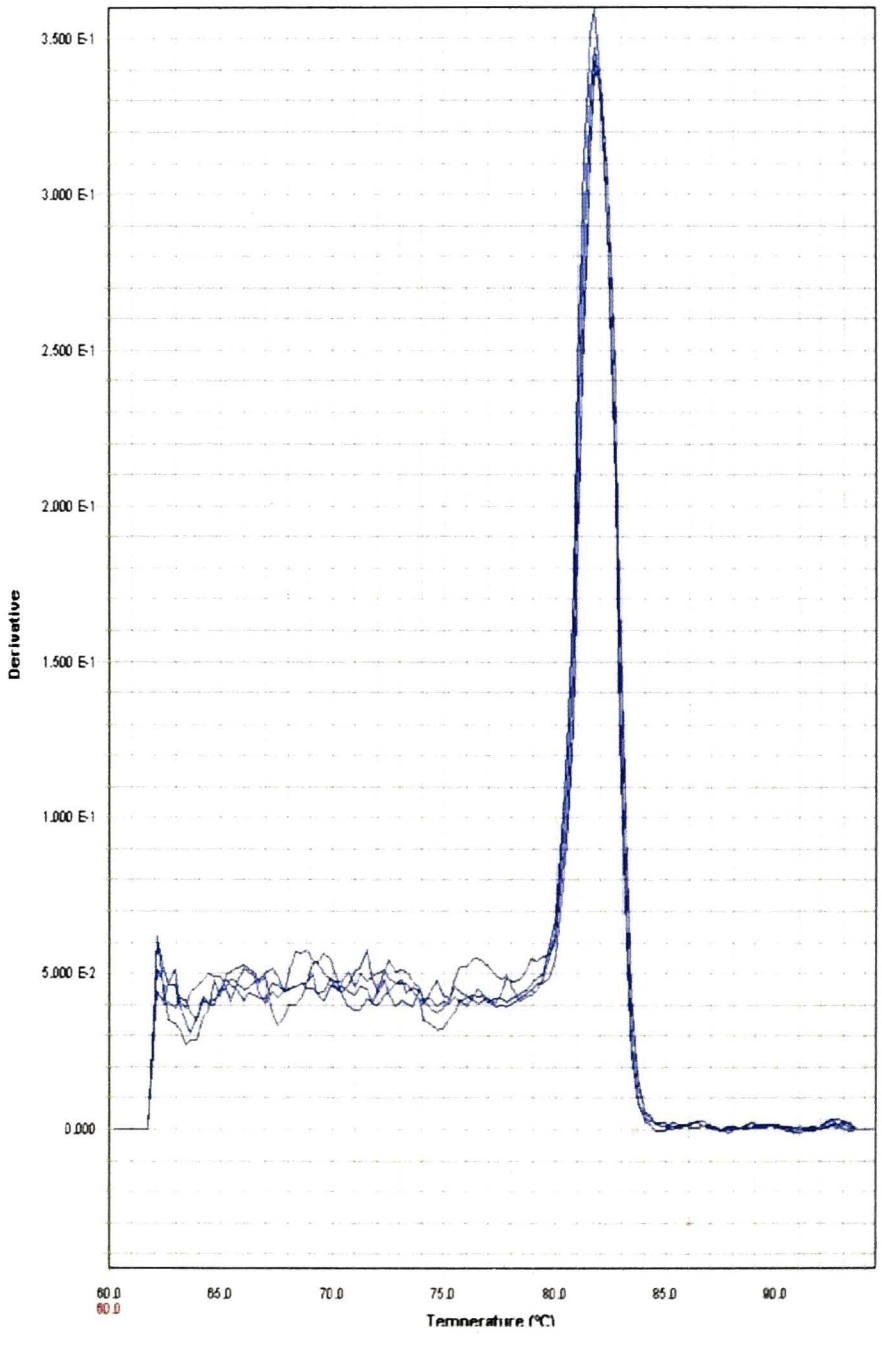
1.400 E-1
9.000 E-2
4.000 E-2
Derivative
80.0
85.0
90.0
Temperature (°C)

IL-23A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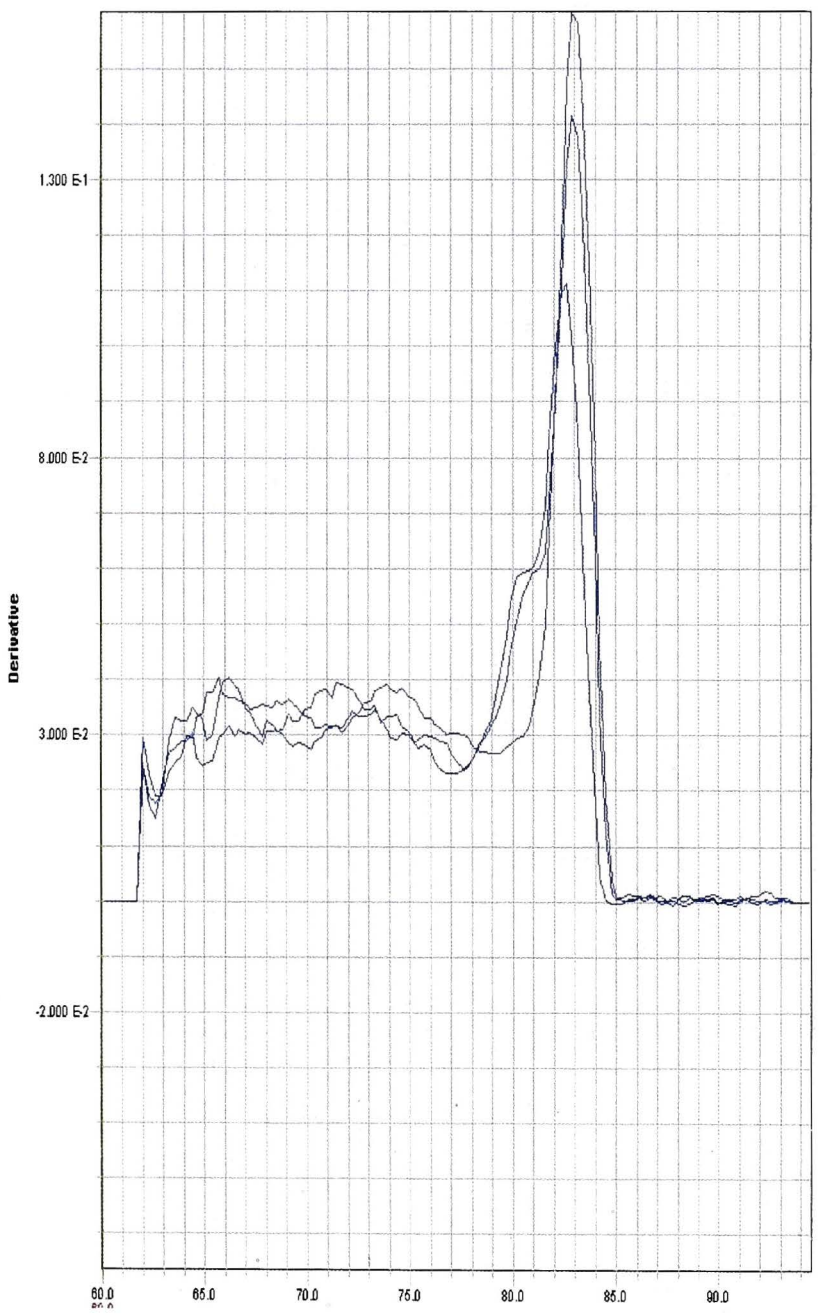
2.500 E-1
2.000 E-1
1.500 E-1
1.000 E-1
5.000 E-2
0.000
Derivative
60.0
65.0
70.0
75.0
80.0
85.0
90.0
Temperature (°C)

T-bet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3.000 E-2
-2.000 E-2
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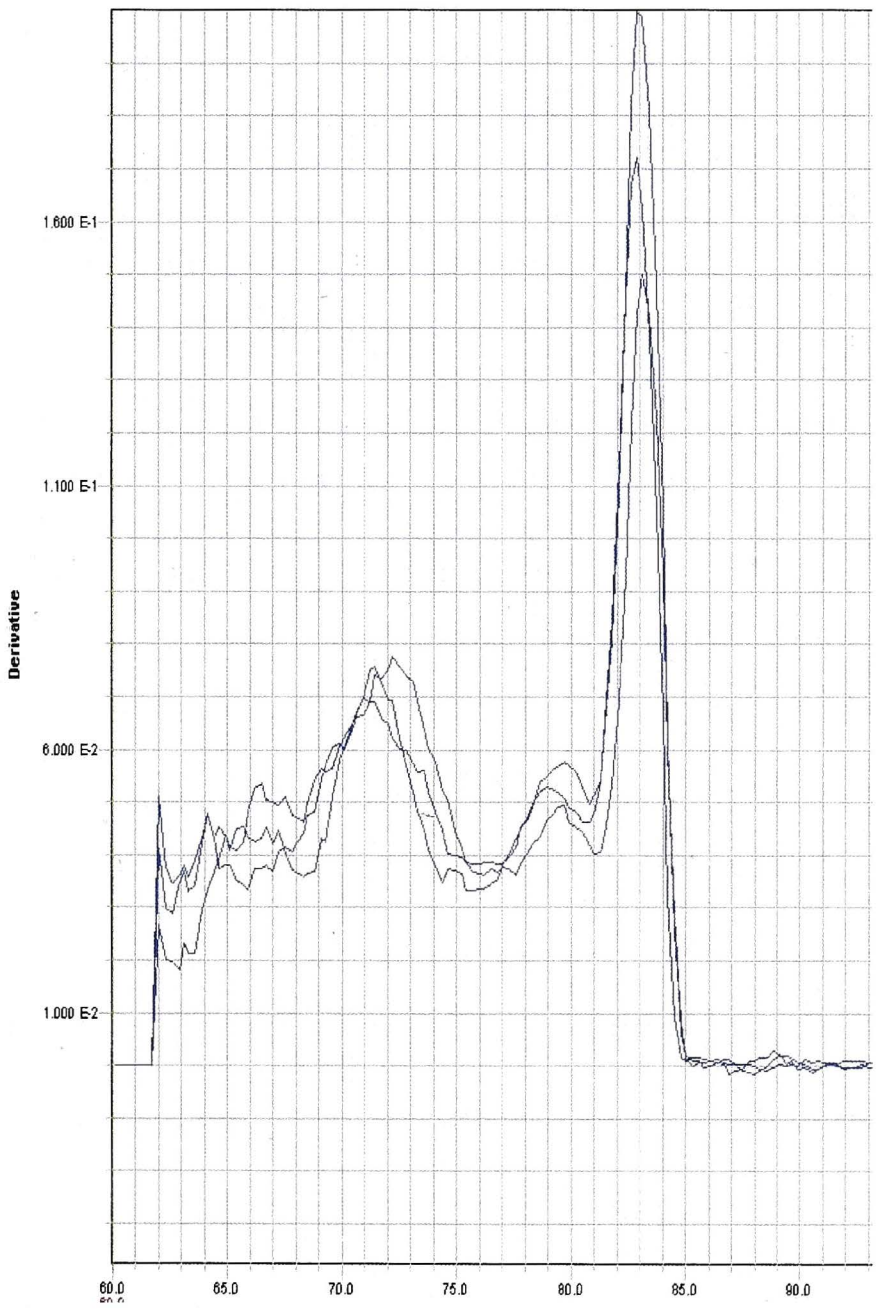
75.0
80.0
85.0
90.0

GATA-3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6.000 E-2
1.000 E-2
Derivative
60.0
65.0
70.0
75.0
80.0
85.0
90.0

ROR-γt

to each well, 20 µl of cDNA synthesizing buffer containing cDNA buffer (1x,QIAGEN), 0.5 mM dNTP (QIAGEN), 125 ng oligo dT (promega, USA), 5U RNasin (Promega), 0.5 nM spermidine (Sigma), 0.1 % triton-X 100 (sigma), 0.125 µl Sensiscript Reverse Transcriptase (Sensiscript RT kit, QIAGEN) and RNase free water was added. $K^dGag_{197-205}$ specific $CD8^+$ T cells (500 cells/well) were sorted in to the above plate using FACS Aria II cell sorter. To synthesize cDNA, the plates were then centrifuged at 2500 rpm for 3 mins to break open the cells and were immediately incubated at 37°C for 110 mins. The plates were then stored at -20°C until further use.

### 2.3.6 Pre-amplification and real time PCR on tetramer specific $CD8^+$ T cells:

To quantify the expression of specific genes in the samples, pre-amplification of cDNA and real time PCR were performed as explained previously in section 2.3.3 and 2.3.4 and the samples were analyzed using SDS software and the arbitrary copy numbers were calculated as before.

## 2.4 Analysis of data and statistics

When plotting ELIspot and ICS data, the unstimulated values were subtracted from the stimulated values except for transcription factor analysis. Experiments were repeated at least three times. To obtain statistics, student's two-tailed paired or unpaired t-Test was performed. Where possible, data from all the repeated experiments were pooled (when plotting graphs) and mean and standard errors of the mean (SEM) and *p* values were calculated respectively. When plotting Real-time data, mRNA copy numbers were calculated from stimulated samples and fold change was calculated by subtracting the stimulated values from unstimulated background. All experiments were repeated twice and data represented are representative of repeat experiments.

**Table 2.7: Primers used in Real time PCR**

| Primer | Sense primer sequence 5'-3' | Anti-sense primer sequence 5'-3' | Reference |
|---|---|---|---|
| IFN-γ | ATGAACGCTACACACTGCATC | AACAGCTGGTGGACCACTC | (Ranasinghe et al., 2007) |
| IL-6 | GAGGATACCACTCCCAACAGACC | AAGTGCATCATCGTTGTTCATACA | (Ranasinghe et al., 2007) |
| IL-17A | TCCAGAAGGCCCTCAGACTA | AGCATCTTCTCGACCCTGAA | (Lexberg et al., 2008) |
| IL-23A | TGGCTGTGCCTAGGAGTAG | AGATGCCCAGCCTGAGTTCT | |
| Granzyme-B | CCACTCTCGACCCTACATGG | GATCCTTCTGTACTGTCAGCT | (Ranasinghe et al., 2007) |
| ROR-γt | TGCAAGACTCATCGACAAGG | AGGGGATTCAACATCAGTGC | (Lexberg et al., 2008) |
| GATA3 | CCTACCGGGTTCGGATGTAAGT | AGTTCGCGCAGGATGTCC | (Lexberg et al., 2008) |
| T-bet | TCCTGCAGTCTCTCCACAAGT | CAGCTGAGTGATCTCTGCGT | (Lexberg et al., 2008) |
| TGF-β | CGTGGAAATCAACGGG | CAGAAGTTGGCATGGT | (Lexberg et al., 2008) |
| L32 | GCTGGAGGTGCTGCTGATGTG | CGTTGGGATTGGTGACTCTGATGG | (Ranasinghe et al., 2007) |

# CHAPTER 3: Elucidate the role of IL-4/IL-13 and STAT6 in modulating IL-17A expressions in HIV-specific $CD8^+$ T cells

### 3.1 Introduction:

Developing vaccines to elicit strong sustained cellular immune responses against HIV-1 has been a challenging task. Even though true correlates of HIV-specific protective immunity in humans are not well understood, it is well established that strong CTL immunity is important for effective HIV-1 virus clearance [1162]. Currently, a body of evidence suggests that durability, breadth of response, avidity and cytokine expression profile of CTL play an important role in HIV-1 protective immunity [163]. Interestingly, several studies have indicated that multifunctional CTL that express cytokines such as IFN-γ, TNF-α and IL-2 are higher in avidity and are a hallmark of protective immunity [139, 146, 164]. In our laboratory, Ranasinghe *et al.* have shown that the magnitude of T cell measured by IFN-γ production and avidity of CTL vary according to the routes of vaccine delivery [132, 144]. They have demonstrated that i) mucosal poxvirus prime-boost immunization (i.n/i.n or i.n/i.m) can generate high avidity $CD8^+$ T cells compared to pure systemic immunization (i.m/i.m), ii) Systemic immunization induce $K^dGag_{197\text{-}205}$ specific $CD8^+$ T cells that express elevated Th2 cytokines IL-13 and IL-4 and these cytokines are inversely correlated with $CD8^+$ T cell avidity [144, 150], iii) Using IL-4-/-, IL-13-/- gene knock out (GKO) mice, they have further substantiated that IL-4 and IL-13 plays an important role in modulating T cell avidity [165].

Recent studies by He *et al.* have shown that IL-4 and IL-13 producing $CD4^+$ T cells negatively regulate IL-17A [69-71]. In another study, Newcomb *et al.* have shown that $CD4^+$Th17 cells, express a functional IL-13Rα1 and IL-13 negatively regulates the expression of IL-17A by these T cells [166]. On the contrary, Harrington *et al.* have suggested that IFN-γ can also negatively regulate Th17 cell differentiation and proliferation [167]. Also, in an IFN-γ deficient mouse model, it has been shown that the expression of IL-17 was enhanced [68, 168]. Interestingly, the expression of IL-17A has been implicated in protective immunity [169, 170]. Therefore, in this study we have investigated how IL-4, IL-13 and IFN-γ are involved in the regulation of IL-17A expression by HIV-specific $CD8^+$ T cells and whether IL-17A plays a direct or indirect role in modulating HIV-specific $CD8^+$ T cell avidity and protective immunity.

### 3.2. Evaluation of IFN-γ and IL-17A expression by HIV specific $CD8^+$ T cells in spleen:

Firstly, intracellular cytokine staining was performed to assess the background splenic expression of IFN-γ and IL-17A by the $CD8^+$ T cells in naive wild type BALB/c and KO mice. Cells were stimulated with $K^dGag_{197\text{-}205}$ peptide for 16-20 hrs, following ICS, samples were run on FACS calibur flow cytometer and 500,000-gated events were acquired. Data were analyzed using Cell Quest Pro analysis software using the gating strategy indicated in Fig 3.1a. The results clearly indicated that in naïve BALB/c, or the GKO mice, there was no IFN-γ or IL-17A expression by the $CD8^+$ T cells following $K^dGag_{197\text{-}205}$ peptide stimulation (Fig 3.1a and 3.1b).

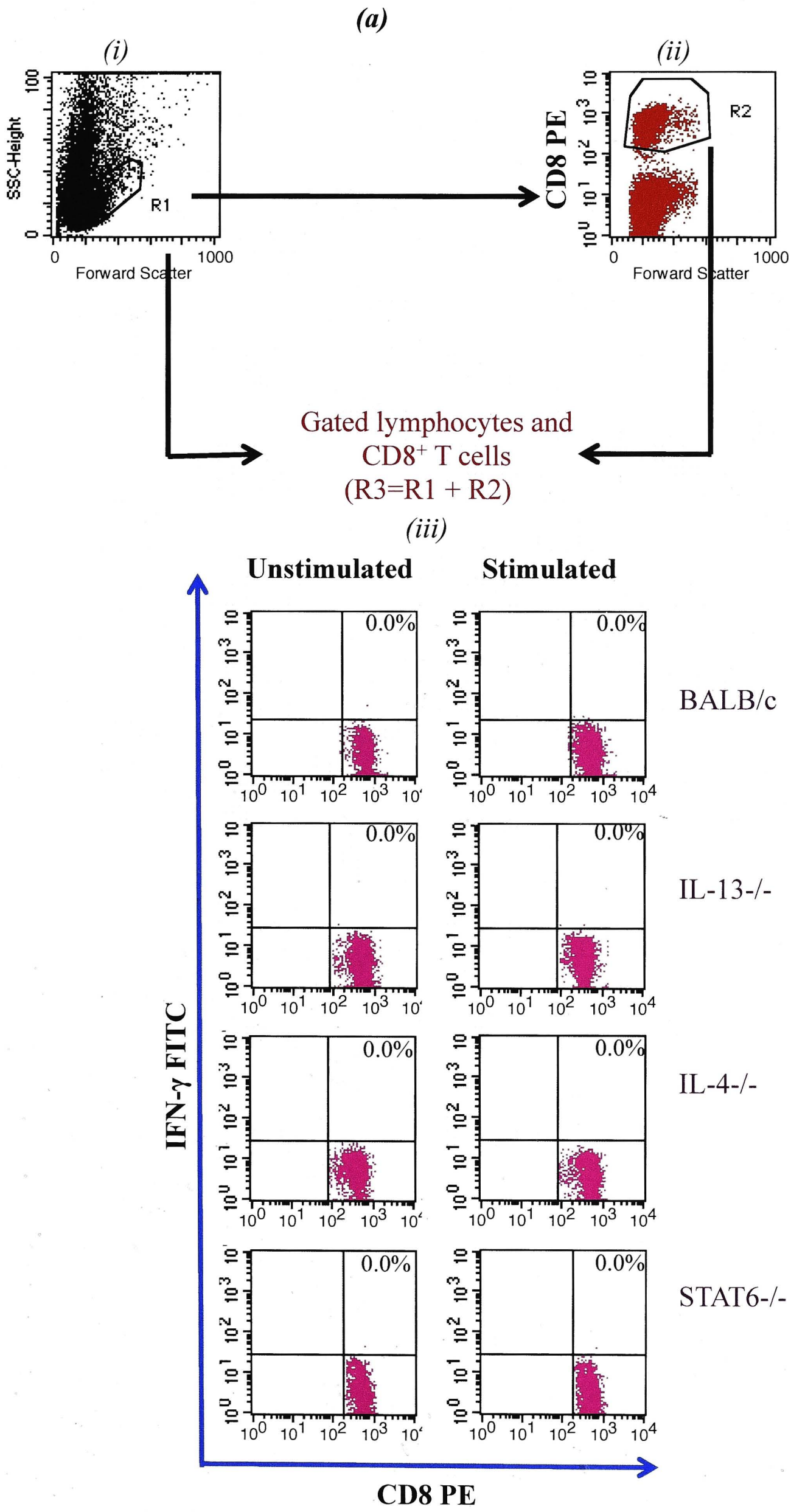
Following vaccination, induction of IFN-γ is often considered as a measurement of effective immunity. Therefore, in this study, firstly, the expression of IFN-γ by $K^dGag_{197\text{-}205}$ specific $CD8^+$ T cells was evaluated. Results indicated that the expression of IFN-γ was higher in IL-4-/- compared to wild type BALB/c mice ($p = 0.020$) (Fig 3.2). In addition, no significant difference in IFN-γ expression was observed between BALB/c, IL-13-/- and STAT6-/- mice, which is consistent with previous finding [165].

Similarly, to determine whether IL-4, IL-13 and STAT6 or IFN-γ down regulate IL-17A expressing $CD8^+$ T cells, IL-4-/-, IL-13-/- and STAT6-/- mice were prime-boost immunized as described previously and the IL-17A expression was evaluated. Results indicated that the expression of IL-17A by the splenic $CD8^+$ T cells in IL-13-/-, IL-4-/- and STAT6-/- mice were significantly higher as compared to the wild type BALB/c control mice (Fig 3.3). The expression profile of IL-17A was IL-4-/- > IL-13-/- > STAT6-/- > BALB/c.

The mulitifunctionality of $CD8^+$ T cells was also investigated to see whether $CD8^+$ T cells produce both IL-17A and IFN-γ. Results indicate that in spleen, $CD8^+$ T cells that expressed elevated levels of IL-17A also expressed IFN-γ in IL-13-/-, IL-4-/- and STAT6-/- mice compare to wild type BALB/c control mice. The expression profile of these double positive $CD8^+$ T cells populations were found to be IL-4 -/- > IL-13-/- > STAT6-/- > BALB/c (Fig 3.4). This clearly indicated that, most of the $CD8^+$ T cells expressing IL-17A in IL-13-/-, IL-4-/- and STAT6-/- mice were also positive for IFN-γ.

**Fig 3.1: Evaluation of IFN-γ and IL-17A expression in naïve BALB/c, IL-13-/-, IL-4-/- and STAT6-/- mice**

*Unimmunized BALB/c, IL-13-/-, IL-4-/- and STAT6-/- (H-$^{2d}$ Background) mice, splenocytes were prepared as described in materials and methods. 4* x *$10^6$ cells were stimulated with $K^dGag_{197-205}$ peptide for 16-20 hrs in the presence of brefeldin-A. IFN-γ (fig 3.1a) and IL-17A (fig 3.1b) expressions were assessed by intracellular cytokine staining. Unstimulated cells from each sample were used as a background controls.* **Gating strategy used in flow-cytometric analysis**: *i) R1 indicate the gated lymphocyte population using forward scatter and side scatter, ii) R2 represent total $CD8^+$ T cells, iii) R3 represent R1 +R2.*


(a)
(i)
(ii)
SSC-Height
100
0
R1
0
1000
Forward Scatter
CD8 PE
R2
Forward Scatter
Gated lymphocytes and
CD8+ T cells
(R3=R1 + R2)
(iii)
Unstimulated
Stimulated
0.0%
BALB/c
IL-13-/-
IL-4-/-
STAT6-/-
IFN-γ FITC
CD8 PE

*(b)*

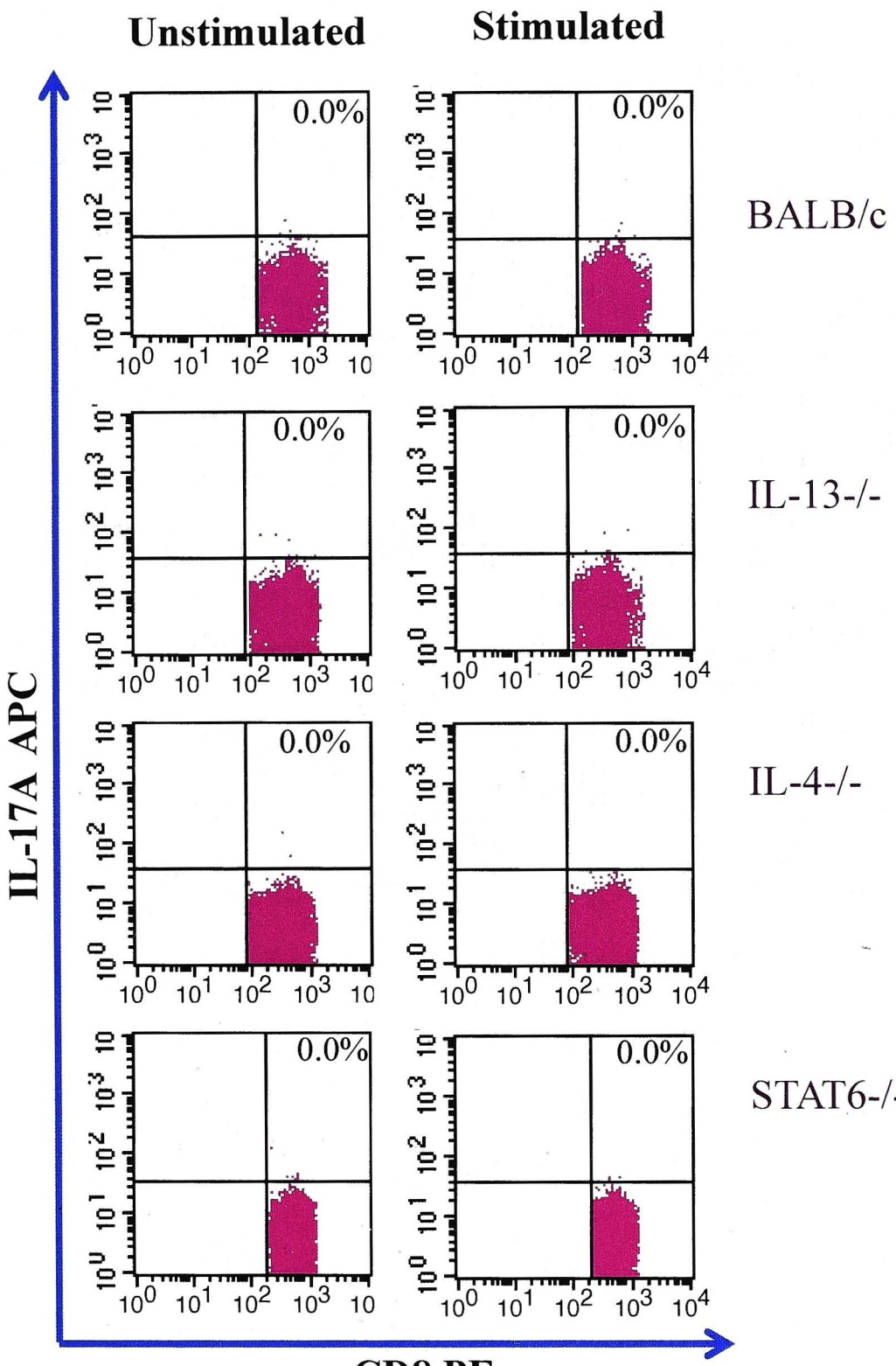
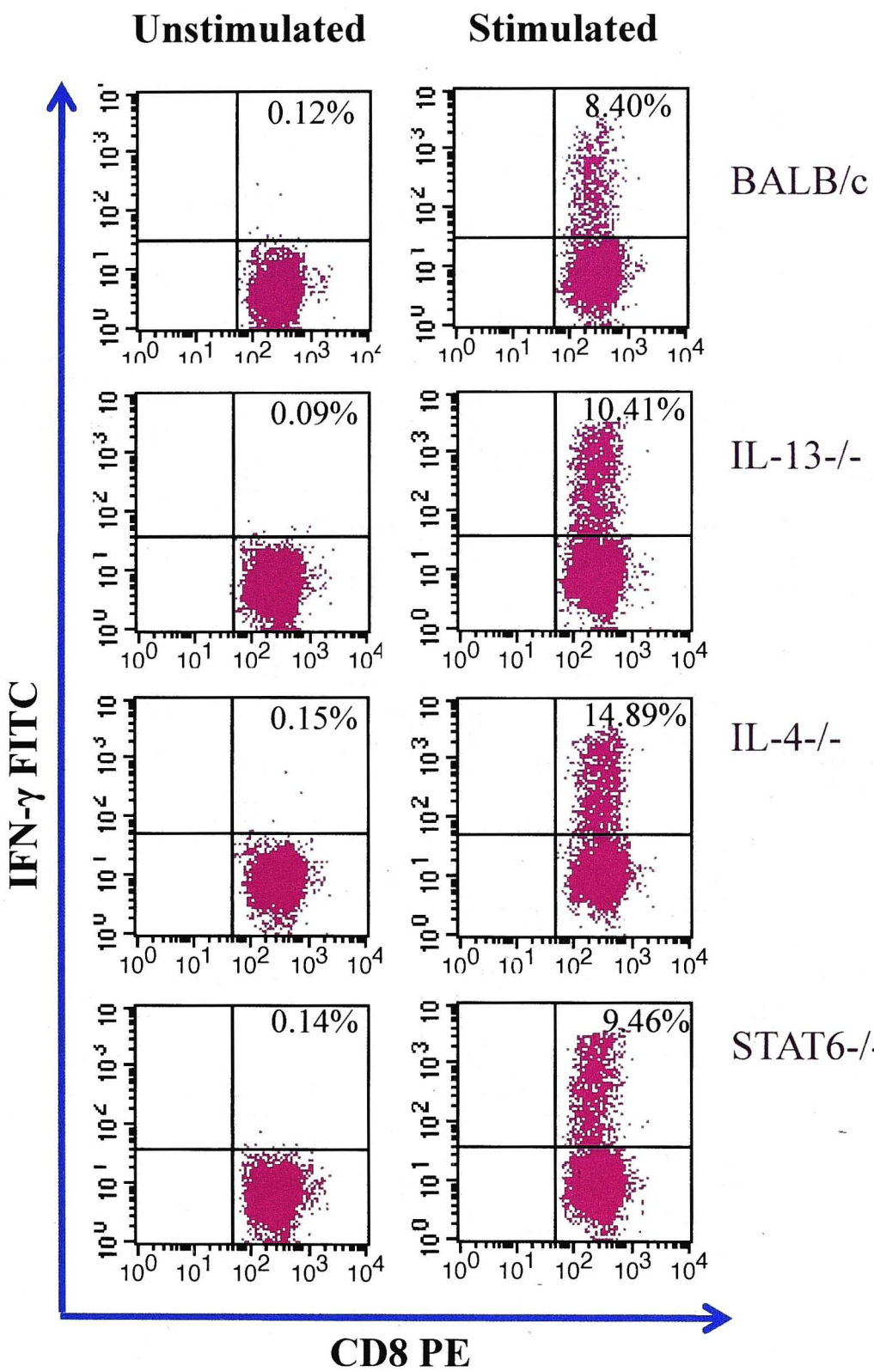

Unstimulated
Stimulated
BALB/c
IL-13-/-
IL-4-/-
STAT6-/-
0.0%
IL-17A APC
CD8 PE

**Fig 3.2: Evaluation of IFN-γ expression by HIV-specific $CD8^+$ T cells following i.n/i.m FPV-HIV/VV-HIV prime-boost immunization**

*BALB/c, IL-13-/-, IL-4-/- and STAT6-/- ($H\text{-}^{2d}$ Background) mice were prime-boost immunized with FPV-HIV/VV-HIV (control vaccine). Splenocytes were prepared as described in materials and methods and 4 x $10^6$ cells were stimulated with $K^dGag_{197\text{-}205}$ peptide for 16-20 hrs in the presence of brefeldin-A to evaluate IFN-γ expression by intracellular cytokine staining. Unstimulated cells from each sample were used as background controls. The FACS plots show representative animals from each group. a) In all FACS plots the upper right quadrant (R3) indicates gates R1+R2 (see fig 3.1) and the numbers indicate the percentage of $CD8^+$ T cells producing IFN-γ. b) The graph indicates the percentage of $CD8^+$ T cells expressing IFN-γ in each group from n=6 mice. In all graphs, unstimulated cells were used as background controls and were subtracted from each sample before plotting the data. Data are representative of four independent experiments and error bars represent standard error of the mean (SEM). p values were calculated using student's two tailed paired t test *p=0.020 (BALB/c Vs. IL-4-/-)*

***(a)***


Unstimulated
Stimulated
0.12%
8.40%
BALB/c
0.09%
10.41%
IL-13-/-
0.15%
14.89%
IL-4-/-
0.14%
9.46%
STAT6-/-
IFN-γ FITC
CD8 PE

***(b)***

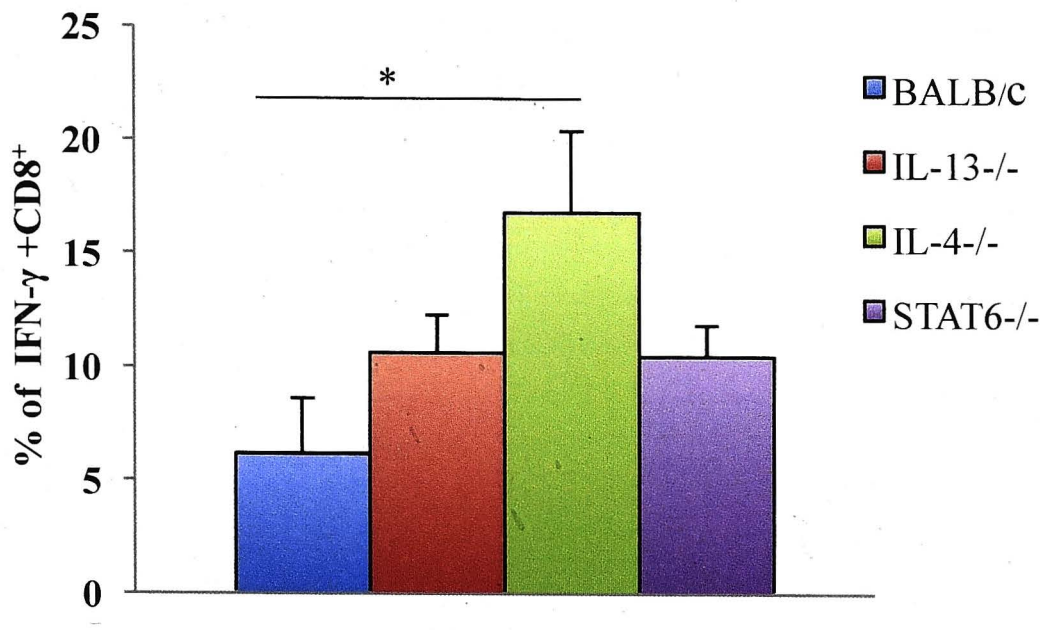
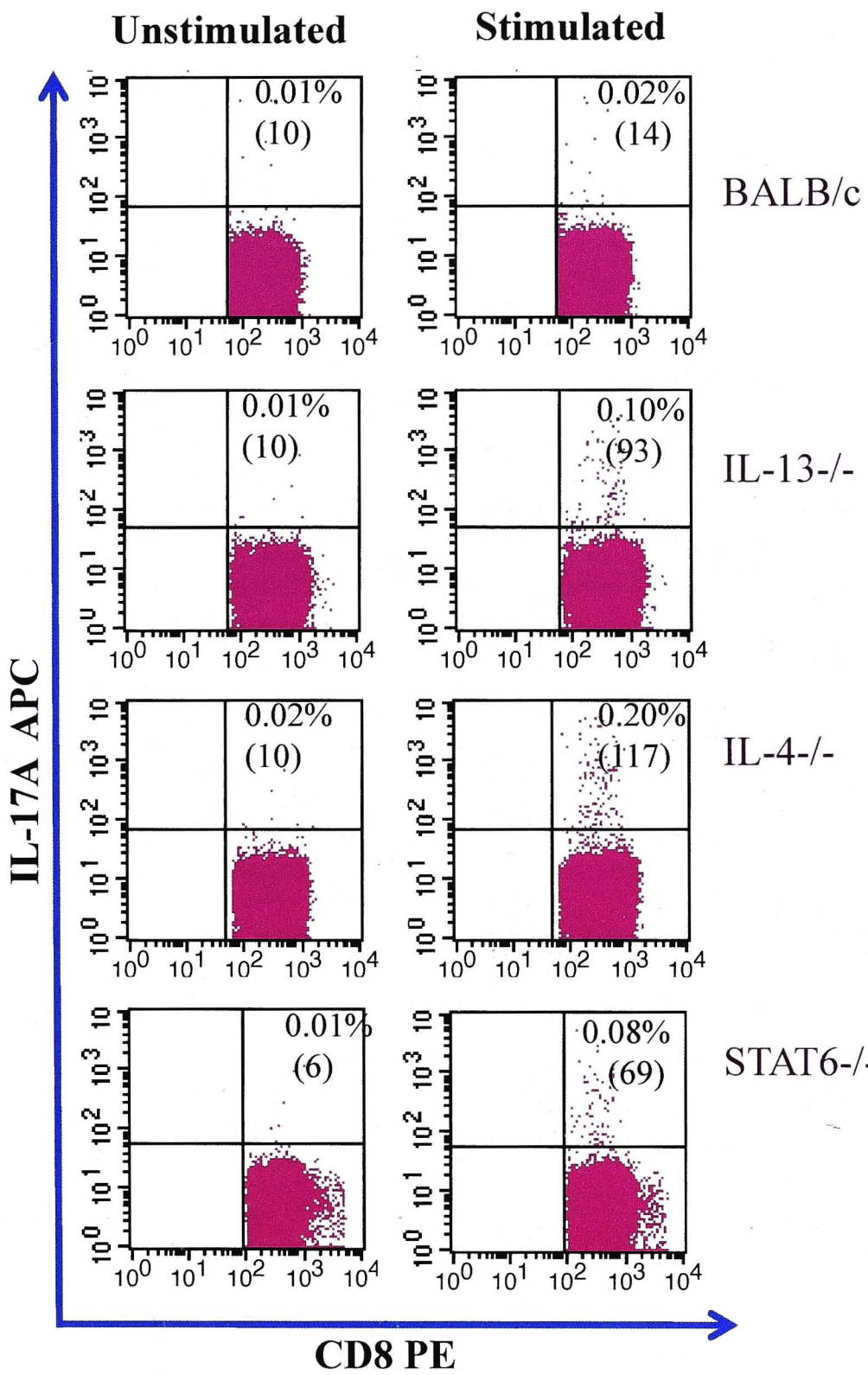

25
20
15
10
5
0
% of IFN-γ +CD8+
*
BALB/c
IL-13-/-
IL-4-/-
STAT6-/-

**Fig 3.3: Evaluation of IL-17A expression by HIV-specific $CD8^+$ T cells following i.n/i.m FPV-HIV/VV-HIV prime-boost immunization**

*BALB/c, IL-13-/-, IL-4-/- and STAT6-/- (H-$^{2d}$ Background) mice were prime-boost immunized with FPV-HIV/VV-HIV (control vaccine). At 14 days post booster immunization, splenocytes were prepared as described in materials and methods and 4 x $10^6$ cells were stimulated with $K^dGag_{197-205}$ peptide for 16-20 hrs in the presence of brefeldin-A and IL-17A expression was evaluated by intracellular cytokine staining. Unstimulated cells from each sample were used as background controls. a) The FACS plots indicate representative animals from each group. In all FACS plots, the upper right quadrant (R3) indicates gates R1+R2 and the numbers indicate IL-17A producing $CD8^+$ T cells as a percentage (top) and the number of events (bottom) within brackets. b) The graph indicates the percentage of $CD8^+$ T cells expressing IL-17A in each of the 4 groups from n=4 mice/group and data represent mean of three independent experiments (n=12 mice/group). In all graphs, unstimulated cells were used as a background controls and were subtracted from each sample before plotting the data. The error bars represent standard error of the mean (SEM). p values were calculated using student's two tailed unpaired t test *p=0.017 (BALB/c Vs. IL-13-/-), **p=0.001 (BALB/c Vs. IL-4-/-) and ***p=0.005 (BALB/c Vs. STAT6-/-).*

*(a)*


Unstimulated
Stimulated
0.01%
(10)
0.02%
(14)
BALB/c
0.01%
(10)
0.10%
(93)
IL-13-/-
0.02%
(10)
0.20%
(117)
IL-4-/-
0.01%
(6)
0.08%
(69)
STAT6-/-
IL-17A APC
CD8 PE

*(b)*

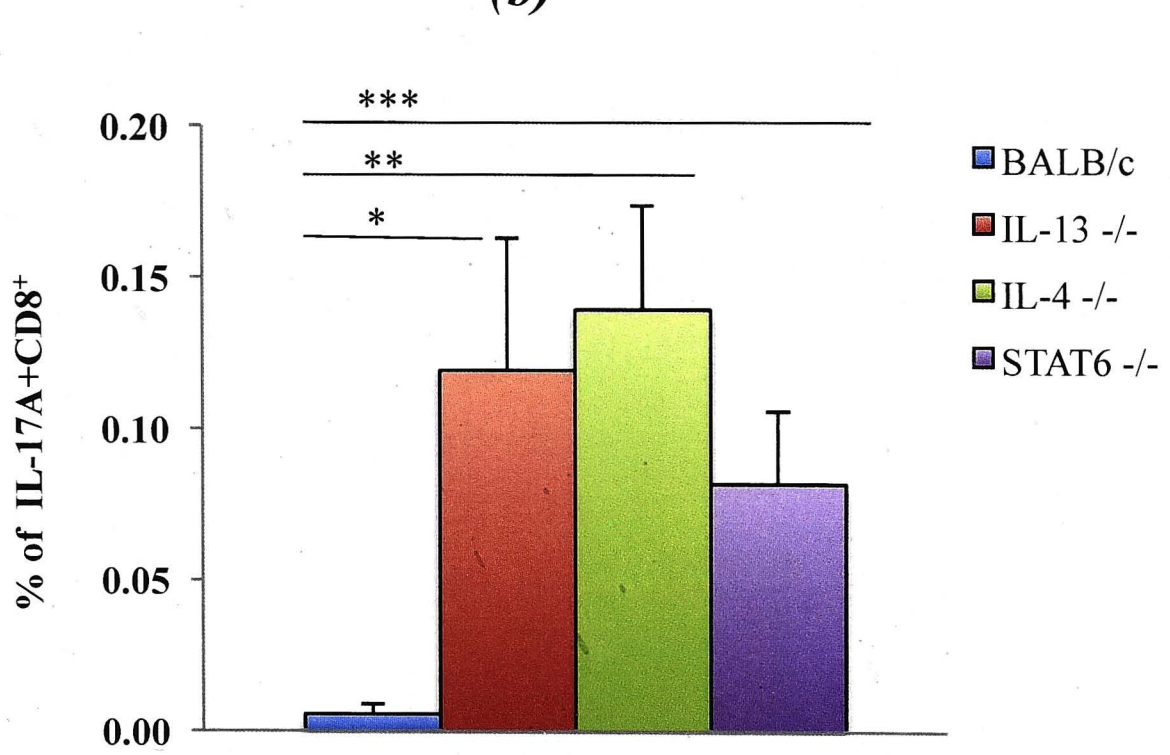
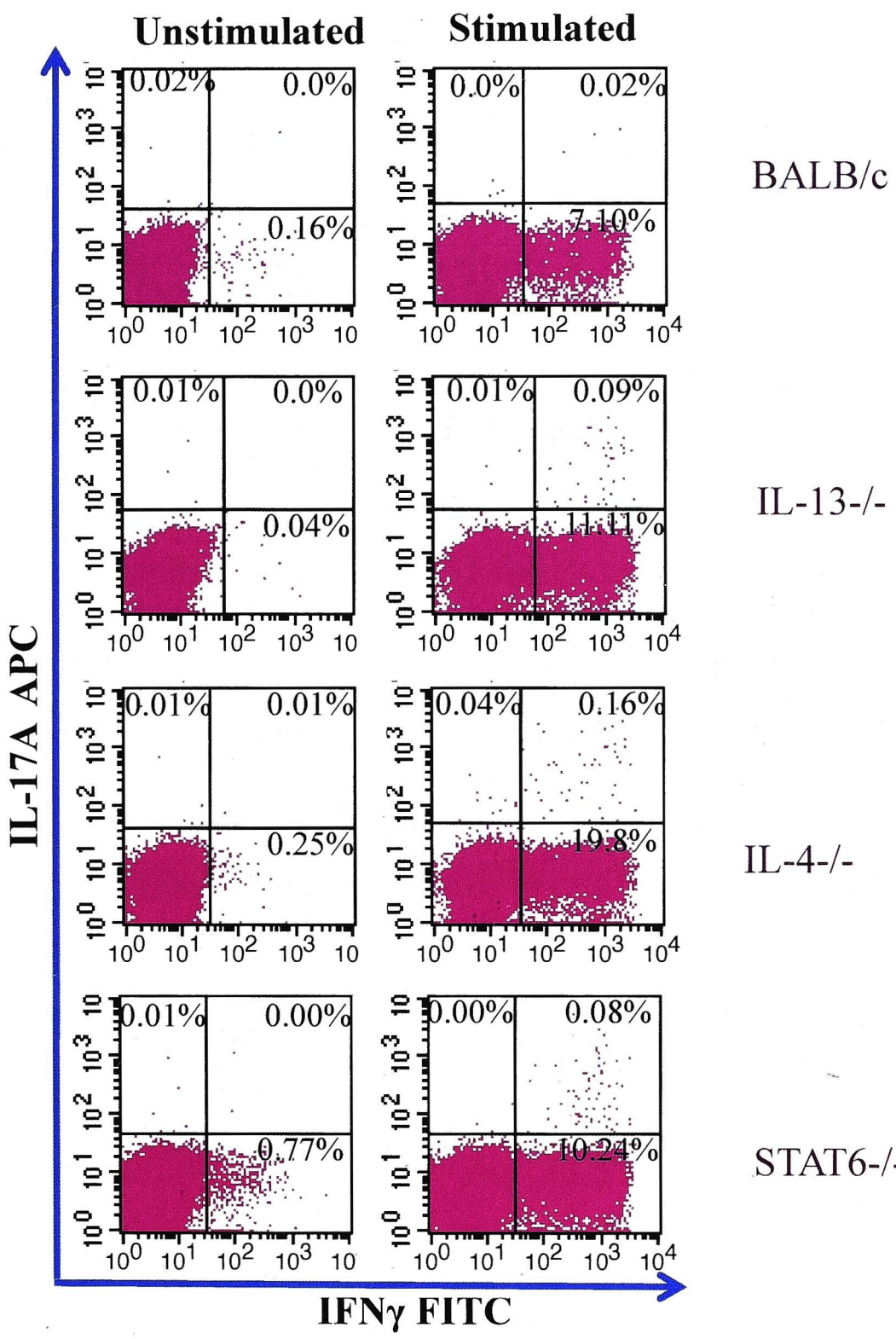

***
**
*
0.20
0.15
0.10
0.05
0.00
% of IL-17A+CD8+
BALB/c
IL-13 -/-
IL-4 -/-
STAT6 -/-

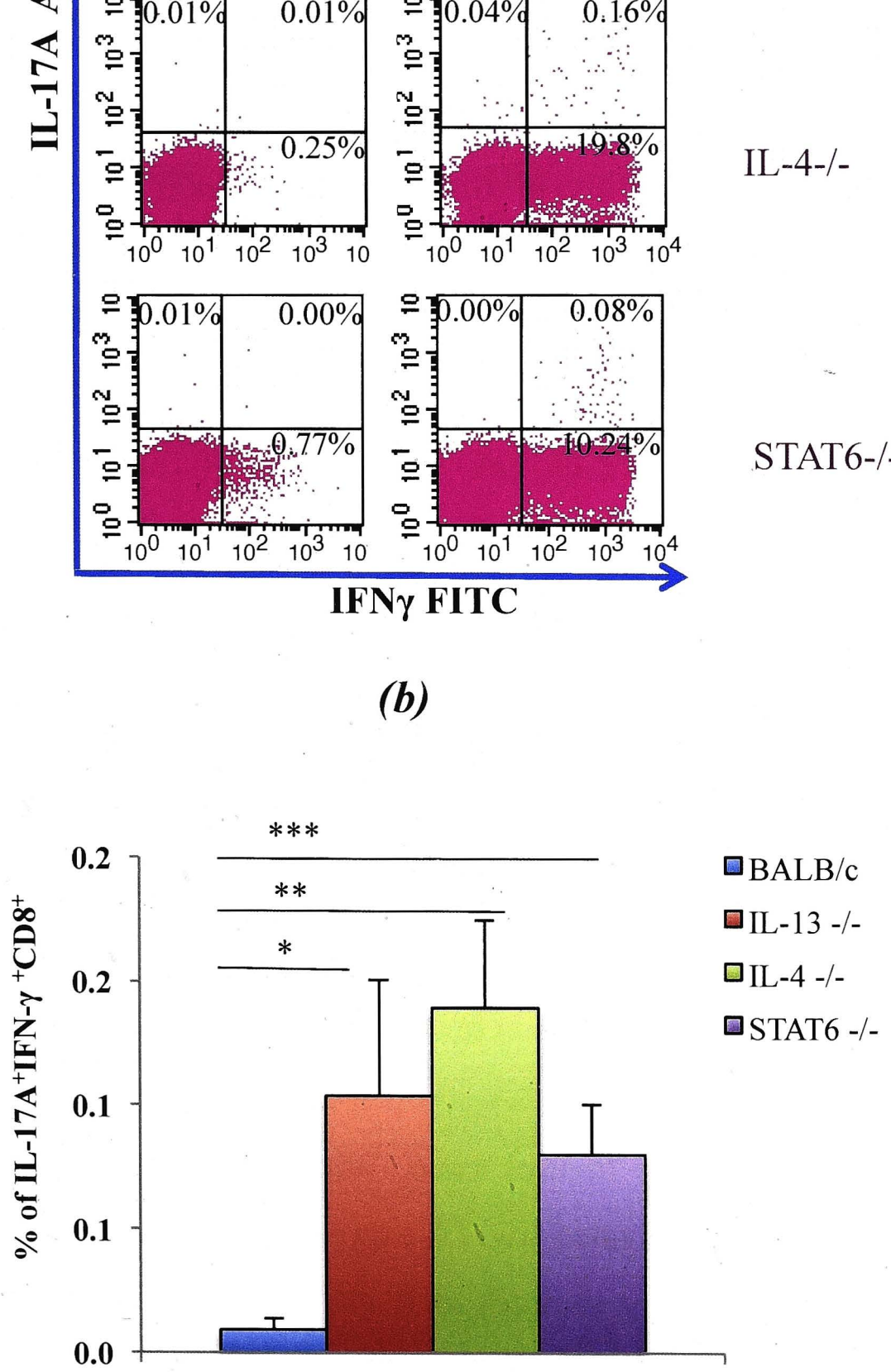
**Fig 3.4: Evaluation of IL-17A$^+$IFN-γ$^+$ (double positive) HIV-specific CD8$^+$ T cells following i.n/i.m FPV-HIV/VV-HIV prime-boost immunization**

*BALB/c, IL-13-/-, IL-4-/- and STAT6-/- (H-$^{2d}$ Background) were prime-boost immunized with FPV-HIV/VV-HIV (control vaccine). At 14 days post booster immunization, splenocytes were prepared as described in materials and methods and 4 x $10^6$ cells were added and stimulated with $K^dGag_{197-205}$ peptide for 16-20 hrs in the presence of brefeldin-A and IL-17 and IFN-γ expression was measured by intracellular cytokine staining. Unstimulated cells from each sample were used as background control. a) The FACS plots indicate representative animals from each group. In all FACS plots, the upper right quadrant (R3) indicates gates R1+R2 and the numbers in the upper right quadrant indicate the percentage of CD8$^+$ T cells producing IL-17A and IFN-γ. Also, the numbers in the upper left quadrant indicate the percentage of CD8$^+$IL-17A$^+$ and the numbers in the lower right quadrant represents the percentage of CD8$^+$IFN-γ$^+$. b) The graph indicates the percentage of CD8$^+$ T cells expressing IL-17A in each of the 4 groups from n=4 mice/group and data represent mean of three independent experiments (n=12 mice/group). Data represents mean of three independent experiments. In all graphs, unstimulated cells were used as a background controls and were subtracted from each sample before plotting the data. The error bars represent standard error of the mean (SEM). p values were calculated using student's two tailed unpaired t test *p=0.058 (BALB/c Vs. IL-13-/-), **p=0.001(BALB/c Vs. IL-4-/-) and ***p=0.004 (BALB/c Vs. STAT6-/-).*

*(a)*


Unstimulated
Stimulated
BALB/c
IL-13-/-
IL-4-/-
STAT6-/-
0.02%
0.0%
0.16%
0.0%
0.02%
7.10%
0.01%
0.0%
0.04%
0.01%
0.09%
11.11%
0.01%
0.01%
0.25%
0.04%
0.16%
19.8%
0.01%
0.00%
0.77%
0.00%
0.08%
10.24%
IL-17A APC
IFNγ FITC

*(b)*


***
**
*
0.2
0.2
0.1
0.1
0.0
% of IL-17A+IFN-γ +CD8+
BALB/c
IL-13 -/-
IL-4 -/-
STAT6 -/-

### 3.3 Evaluation of IL-17A expression by $K^dGag_{197-205}$ specific splenic $CD8^+$ T cells using ELIspot:

Next, the total number of $CD8^+$ T cells producing IL-17A was also evaluated using ELIspot. As the number of $CD8^+$ T cells expressing IL-17 was relatively low, $CD8^+$ T cells were negatively enriched and IL-17A expression was further evaluated. Results showed the expression of IL-17 SFU were higher in IL-13-/-, IL-4-/- and STAT6-/- animals compared to wild type BALB/c control mice (Fig 3.5a). These results were highly consistent with the number of IL-17A per $10^6$ $CD8^+$ T cells measured by intracellular cytokine staining (Fig 3.5b).

### 3.4 Evaluation of IL-17A expression kinetics at different time intervals following $K^dGag_{197-205}$ peptide stimulation:

It has been previously shown in our laboratory that different cytokines are expressed at different time intervals following antigenic peptide stimulation [144]. Therefore, the expression kinetics of IL-17A was evaluated at 6 hrs and 16 hrs following $K^dGag_{197-205}$ peptide stimulation of splenocytes using intracellular cytokine staining. Results clearly indicated that there was no significant difference in the expression of IL-17A at 6 hrs or 16 hrs post peptide stimulation (Fig 3.6). Also, both KO and BALB/c mice showed similar expression profiles. Interestingly, the IL-17A expression kinetic pattern was similar to that observed for IFN-γ (Stephanie Day Ph.D. thesis 2008).

### 3.5 Evaluation of IFN-γ and IL-17A expression in lung:

Previously, i.n/i.m immunizations have been shown to generate both mucosal and systemic immunity [132]. Also, studies have shown that following viral infections, $CD4^+$ and $CD8^+$ T cells express elevated IL-17A in the lung [19]. Therefore, the expressions of IFN-γ and IL-17A by $K^dGag_{197-205}$ specific $CD8^+$ T cells in the lung were evaluated by intracellular cytokine staining. Results indicated that there was no significant difference in the IFN-γ expression levels between IL-13-/- and wild type (Fig 3.7). Even though, much greater IFN-γ expression was observed in IL-4-/- and STAT6 -/- compared to wild type BALB/c control mice. When the IL-17A expression was examined, unlike BALB/c mice, elevated IL-17A expression was detected by $K^dGag_{197-205}$ specific $CD8^+$ T cells in the lung samples of IL-4-/-, IL-13-/-, and STAT6-/- mice (Fig 3.8).

Recent studies have shown that, $CD8^+$ T cells expressing IFN-γ and IL-17A are implicated in protective immunity [171]. Therefore, the HIV-specific $CD8^+$ T cells that express both IL-17A and IFN-γ were evaluated in the lung following i.n FPV-HIV/i.m VV-HIV prime-boost immunization. Data indicated that similar to the spleen responses, the expressions of IL-17A and IFN-γ double positive $CD8^+$ T cells were elevated in IL-13-/-, IL-4-/- and STAT6-/- mice compared to the wild type control mice (Fig 3.9).

To further confirm the ICS data, IL-17A ELIspot was also performed. Unlike the spleen, due to a small sample size $CD8^+$ T cells were not enriched for the lung. In BALB/c and KO mice, an enhanced number of IL-17 SFU were observed in both stimulated and unstimulated samples (Fig 3.10a, 3.10b). However, IL-4-/- mice exhibited greater numbers of IL-17A SFU following $K^dGag_{197\text{-}205}$ stimulation compared to other KO mice. Interestingly, the ELIspot data did not correlate with ICS data. These differences could be due to non-enriched lung cells used in these assays.

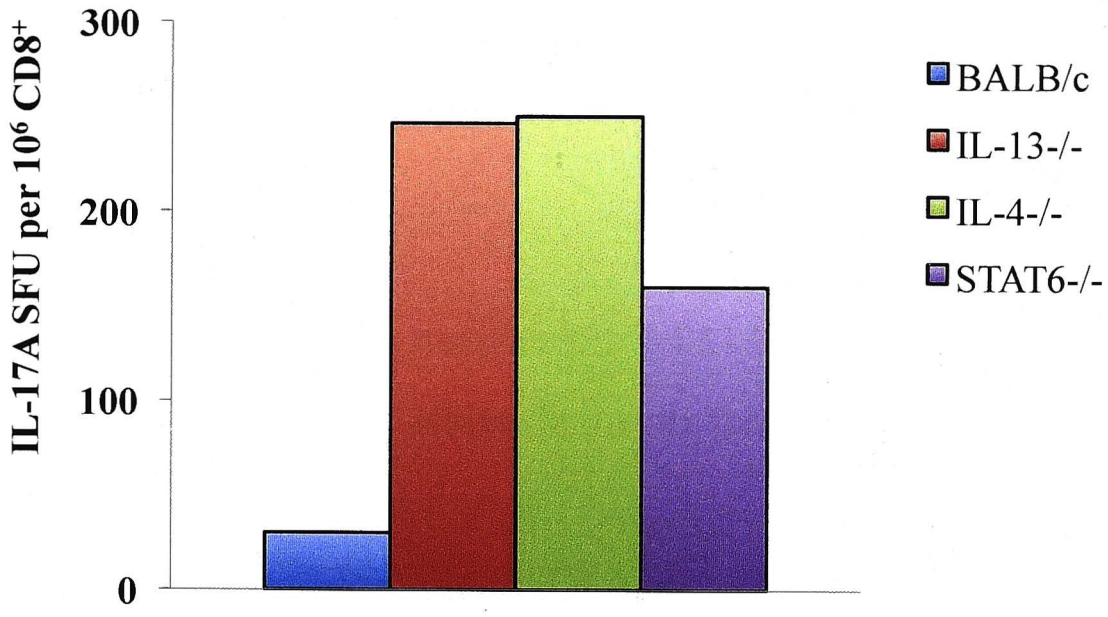
### 3.6 Evaluation of transcription factor ROR-γt protein following prime-boost immunization:

It is now established that the transcription factor, ROR-γt plays an important role in regulating IL-17A expression in Th17 cells [57, 172]. In this study, the expression of ROR-γt from BALB/c, IL-13-/-, IL-4-/- and STAT6-/- (by HIV-specific $CD8^+$T cells) were evaluated at 6 hrs following peptide stimulation. Interestingly, no significant difference in ROR-γt protein expression was observed between stimulated and unstimulated $CD8^+$ T cells obtained from BALB/c and KO mice (Fig 3.11). However, IL-4-/- and STAT6-/- mice showed much greater background ROR-γt expression compared to BALB/c and IL-13-/-mice. The ROR-γt protein profile were IL-4-/- > STAT6-/- > IL-13-/- = BALB/c suggesting IL-4 and STAT6 play role in modulating ROR-γt expression in BALB/c mice.

**Fig 3.5: Evaluation of IL-17A expression by HIV-specific $CD8^+$ T cells using ELIspot**

*a) BALB/c, IL-13-/-, IL-4-/- and STAT6-/- (H-$^{2d}$ Background) mice were prime-boost immunized with FPV-HIV/VV-HIV (control vaccine). At 14 days post-booster immunization, splenocytes were prepared as described in materials and methods. $CD8^+$ T cells from pooled spleen cells were negatively enriched and 4 x $10^6$ cells were stimulated with $K^dGag_{197-205}$ peptide for 24 hrs and IL-17A expression was measured by ELIspot. The graphs indicate the total number IL-17A SFU per $10^6$ $CD8^+$T cells compared to the number of IL-17A cells per $10^6$ $CD8^+$ T cells measured by ICS. b) The error bars represent standard error of the mean (SEM). p values were calculated using student's two tailed unpaired t test *p=0.030 (BALB/c vs. IL-13-/-), **p=0.001 (BALB/c Vs. IL-4-/-) and ***p=0.004 (BALB/c Vs. STAT6-/-).*

***(a) ELIspot***




***(b) ICS as $10^6$ CD8 T cells***

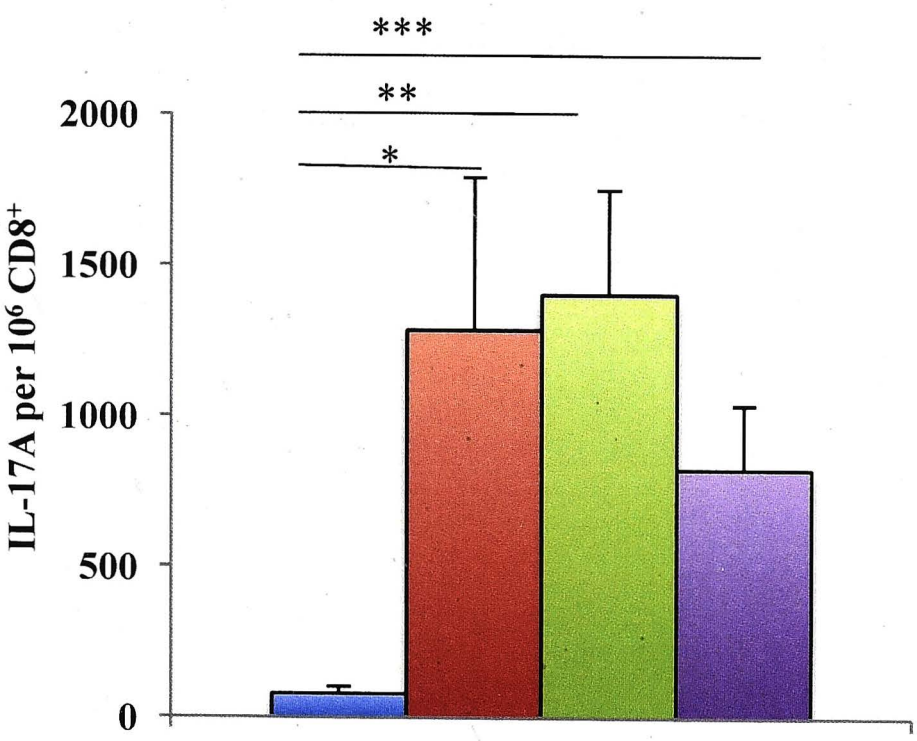


**Fig 3.6: Evaluation of IL-17A expression kinetics following in/i.m FPV-HIV/VV-HIV prime-boost immunization**

*BALB/c, IL-13-/-, IL-4-/- and STAT6-/- (H-$^{2d}$ Background) mice were prime-boost immunized with FPV-HIV/VV-HIV. At 14 days post booster immunization, splenocytes were prepared and 4 x $10^6$ cells were stimulated with $K^dGag_{197-205}$ peptide for 6 hrs and 16 hrs in the presence of brefeldin-A and the expression of IL-17A by $CD8^+$T cells were evaluated by intracellular cytokine staining. In all graphs, unstimulated cells were used as a background controls and were subtracted before plotting the data. The graph indicates the percentage of $CD8^+$ T cells expressing IL-17A at different time intervals.*

***(a)***

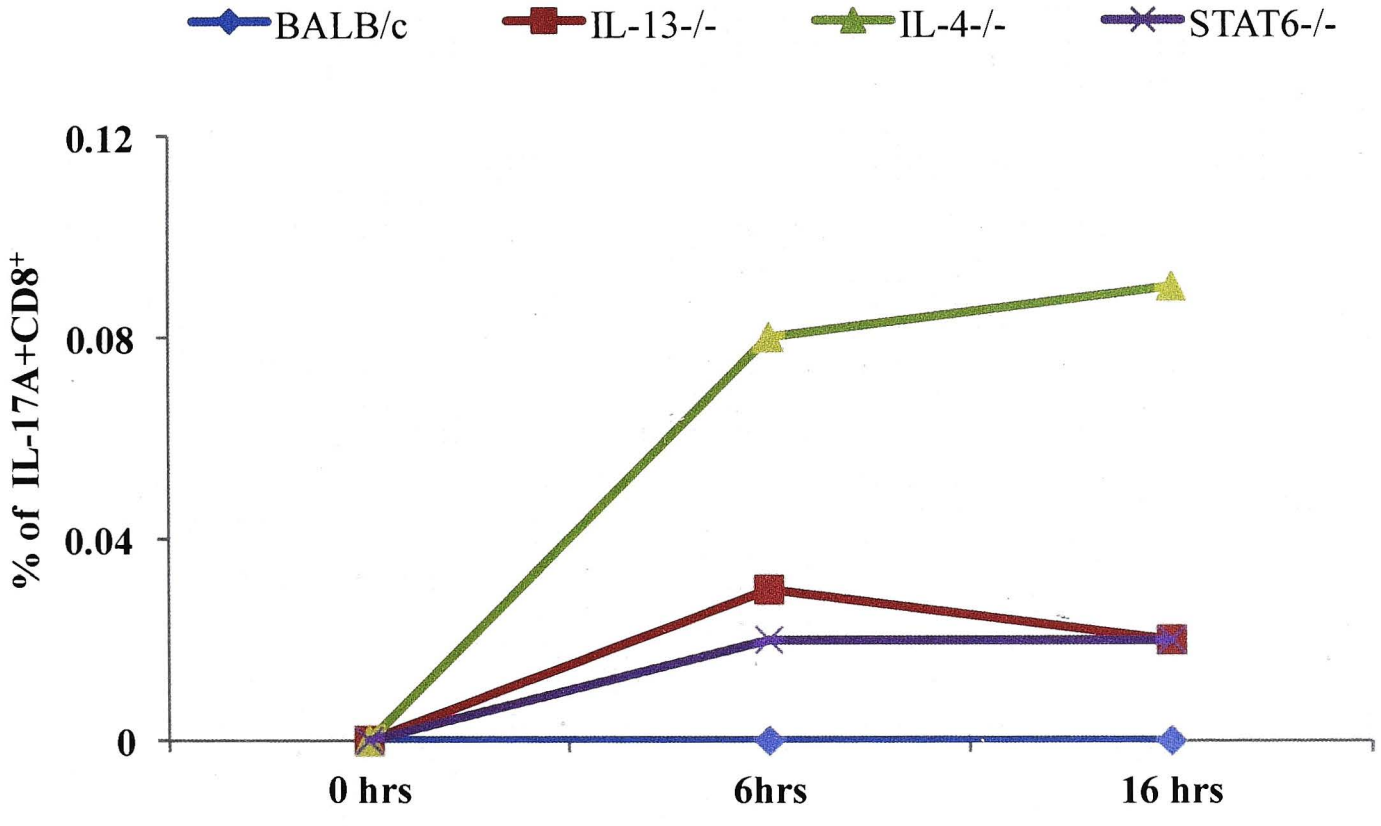
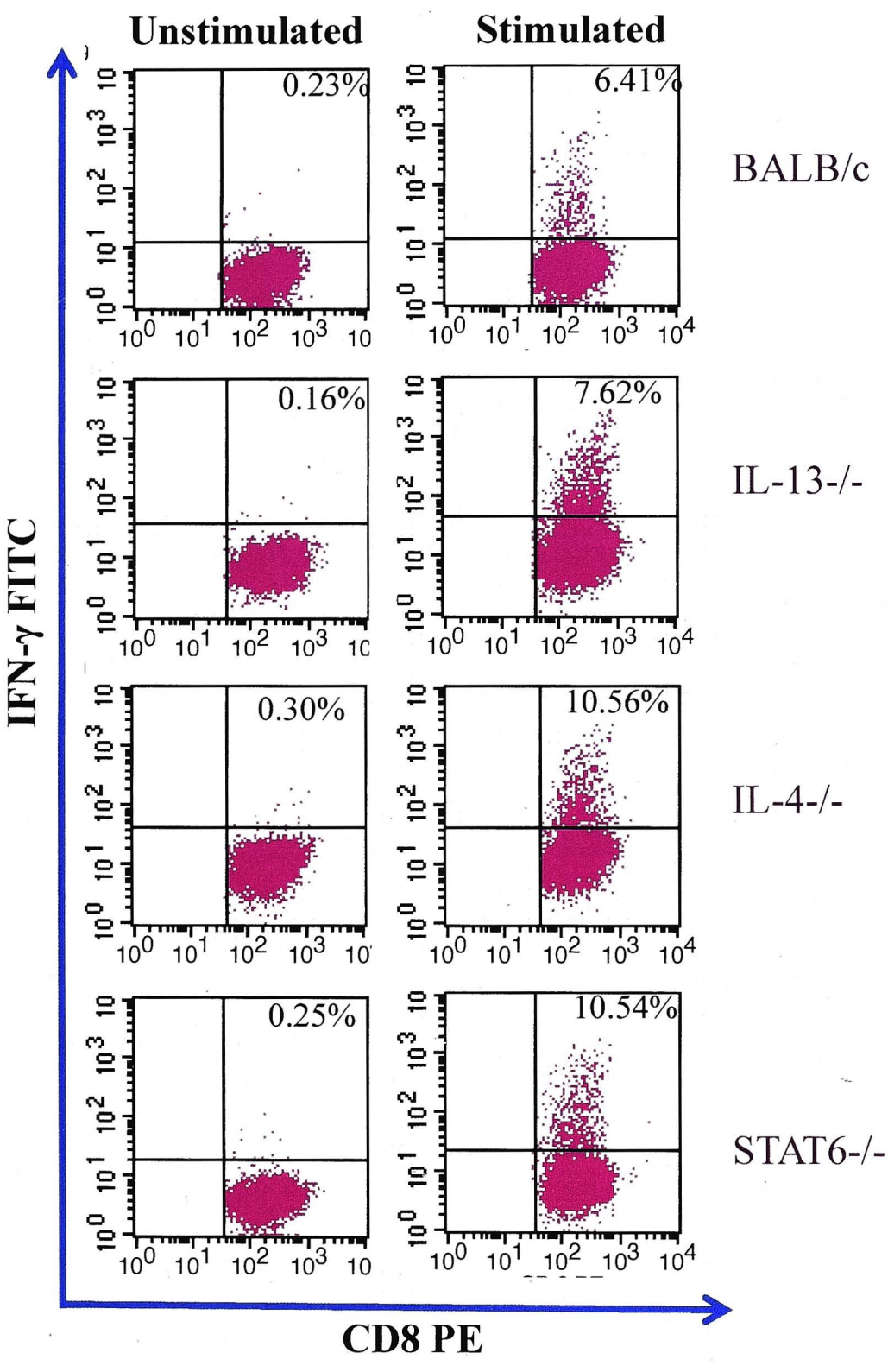

BALB/c
IL-13-/-
IL-4-/-
STAT6-/-
0.12
0.08
0.04
0
% of IL-17A+CD8+
0 hrs
6hrs
16 hrs

**Fig 3.7: Evaluation of lung specific IFN-γ expression by HIV-specific $CD8^+$ T cells following i.n/i.m FPV-HIV/VV-HIV prime-boost immunization**

*BALB/c, IL-13-/-, IL-4-/- and STAT6-/- ($H\text{-}^{2d}$ Background) mice (n=4) were prime-boost immunized with FPV-HIV/VV-HIV (control vaccine). At 14 days post booster immunization, lung cells were prepared as described in materials and methods. 4 x $10^6$ cells were stimulated with $K^dGag_{197\text{-}205}$ peptide for 16-20 hrs in the presence of brefeldin-A and IFN-γ expression was evaluated by intracellular cytokine staining. a) The FACS plots indicate pooled samples from each group. In all FACS plots, the upper right quadrant (R3) indicates gates R1+R2 and the numbers in the quadrant indicate the percentage of $CD8^+$IFN-γ. b) The graph indicates the percentage of $CD8^+$ T cells expressing IFN-γ from each group as a pooled value from n=4 mice/group. In all graphs, unstimulated cells were used as a background controls and were subtracted from each sample before plotting the data. Data are representative of two independent experiments.*

*(a)*


Unstimulated
Stimulated
0.23%
6.41%
BALB/c
0.16%
7.62%
IL-13-/-
0.30%
10.56%
IL-4-/-
0.25%
10.54%
STAT6-/-
IFN-γ FITC
CD8 PE

*(b)*

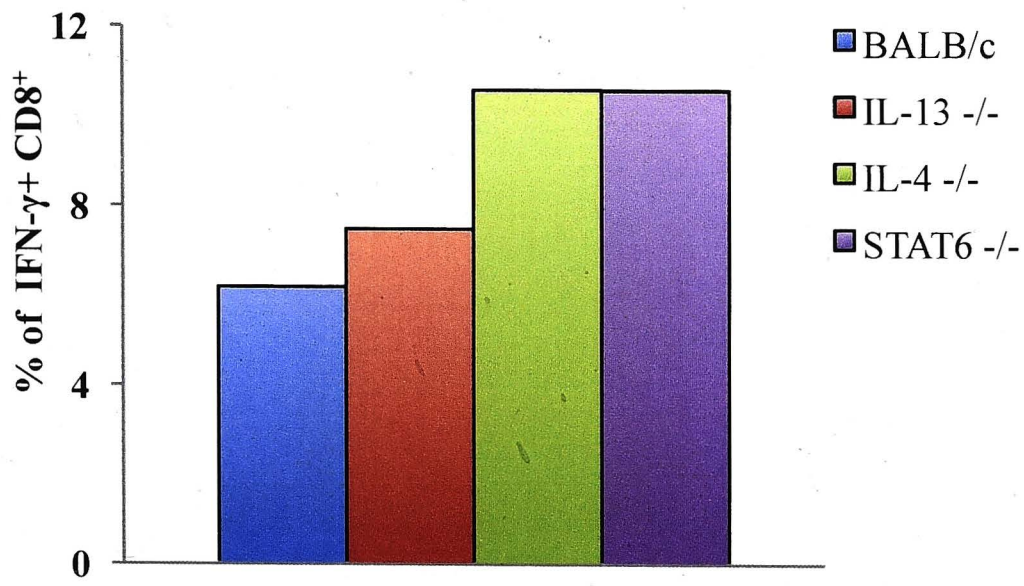
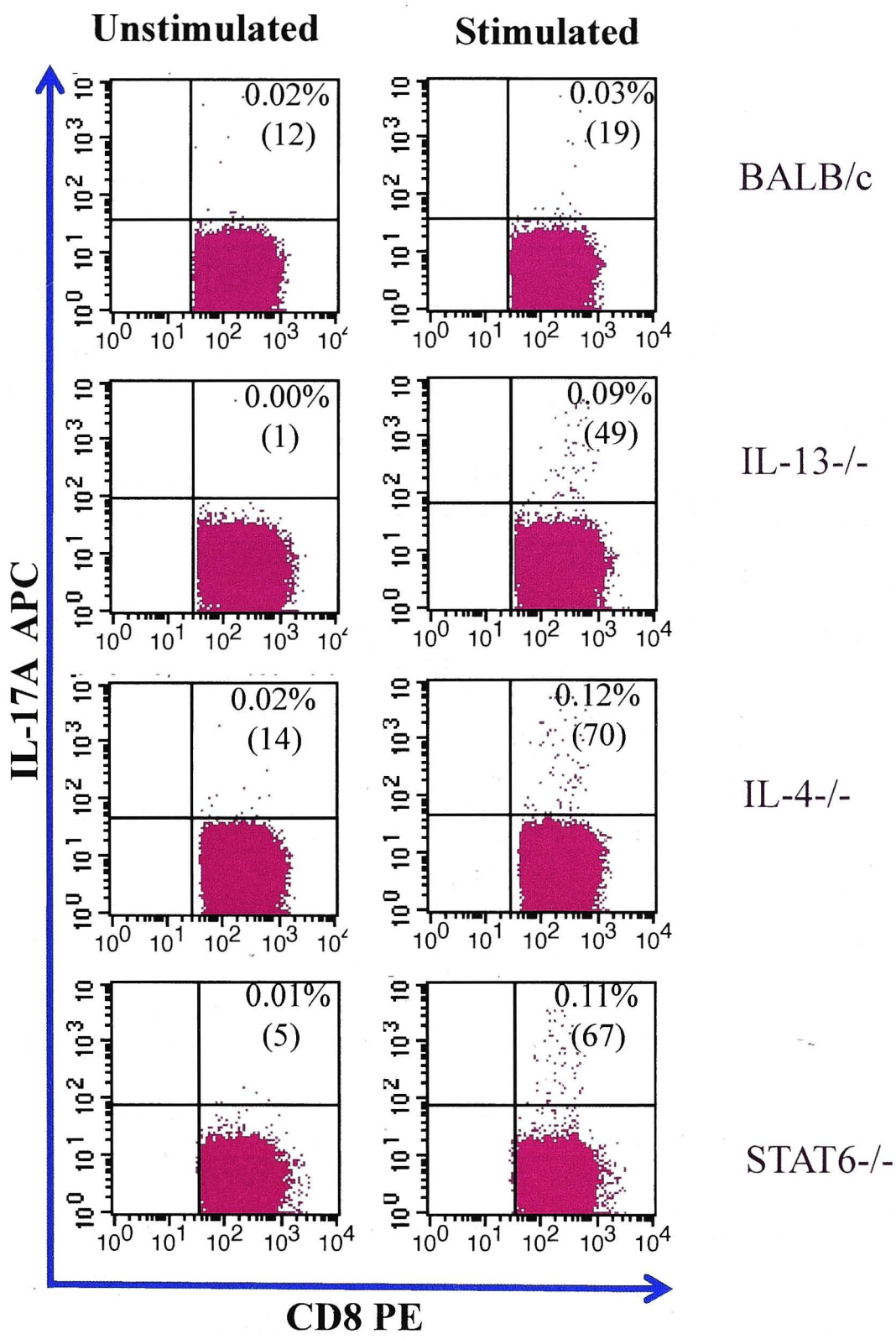

% of IFN-γ+ CD8+
12
8
4
0
BALB/c
IL-13 -/-
IL-4 -/-
STAT6 -/-

**Fig 3.8: Evaluation of lung IL-17A expression by HIV-specific $CD8^+$ T cells following i.n/i.m FPV-HIV/VV-HIV prime-boost immunization**

*BALB/c, IL-13-/-, IL-4-/- and STAT6-/- (H-$^{2d}$ Background) mice (n=4) were prime-boost immunized with FPV-HIV/VV-HIV (control vaccine). At 14 days post booster immunization, lung cells were prepared as described in materials and methods. 4 x $10^6$ cells were stimulated with $K^dGag_{197-205}$ peptide for 16-20 hrs in the presence of brefeldin-A and IL-17A expression was evaluated by intracellular cytokine staining. a) The FACS plots indicate pooled samples from each group. In all FACS plots, the upper right quadrant (R3) indicates R1+R2 and the numbers indicate IL-17A producing $CD8^+$ T cells as a percentage (top) and also the gated number of events (bottom) within brackets. b) The graph indicates the percentage of HIV-specific $CD8^+$ T cells expressing IL-17A from each group as a pooled value from n=4 mice/group. In all graphs, unstimulated cells were used as a background controls and were subtracted from each sample before plotting the data. Data are representative of two independent experiments.*

*(a)*


Unstimulated
Stimulated
0.02%
(12)
0.03%
(19)
BALB/c
0.00%
(1)
0.09%
(49)
IL-13-/-
0.02%
(14)
0.12%
(70)
IL-4-/-
0.01%
(5)
0.11%
(67)
STAT6-/-
IL-17A APC
CD8 PE

*(b)*

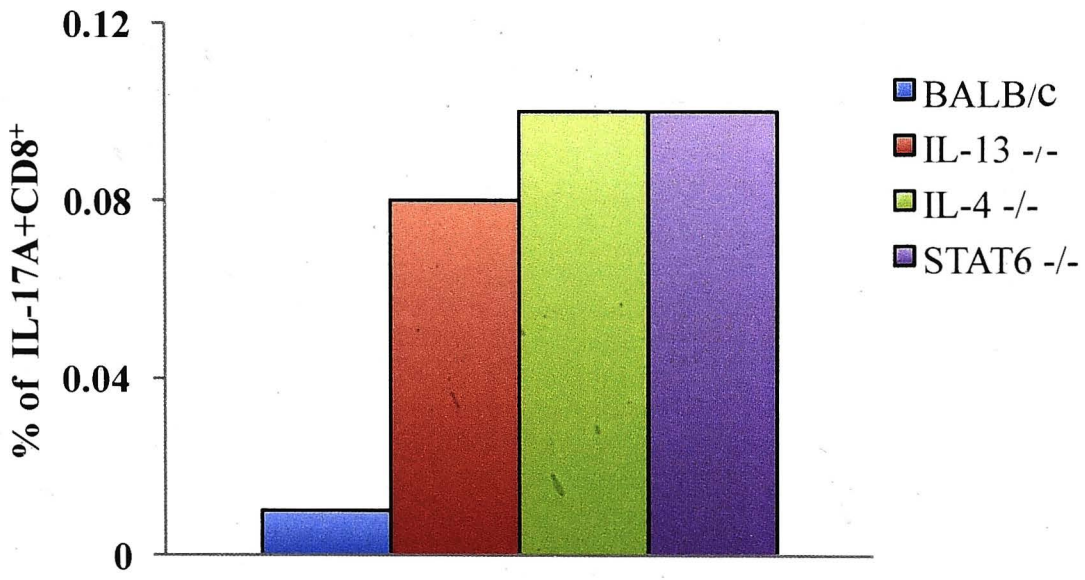
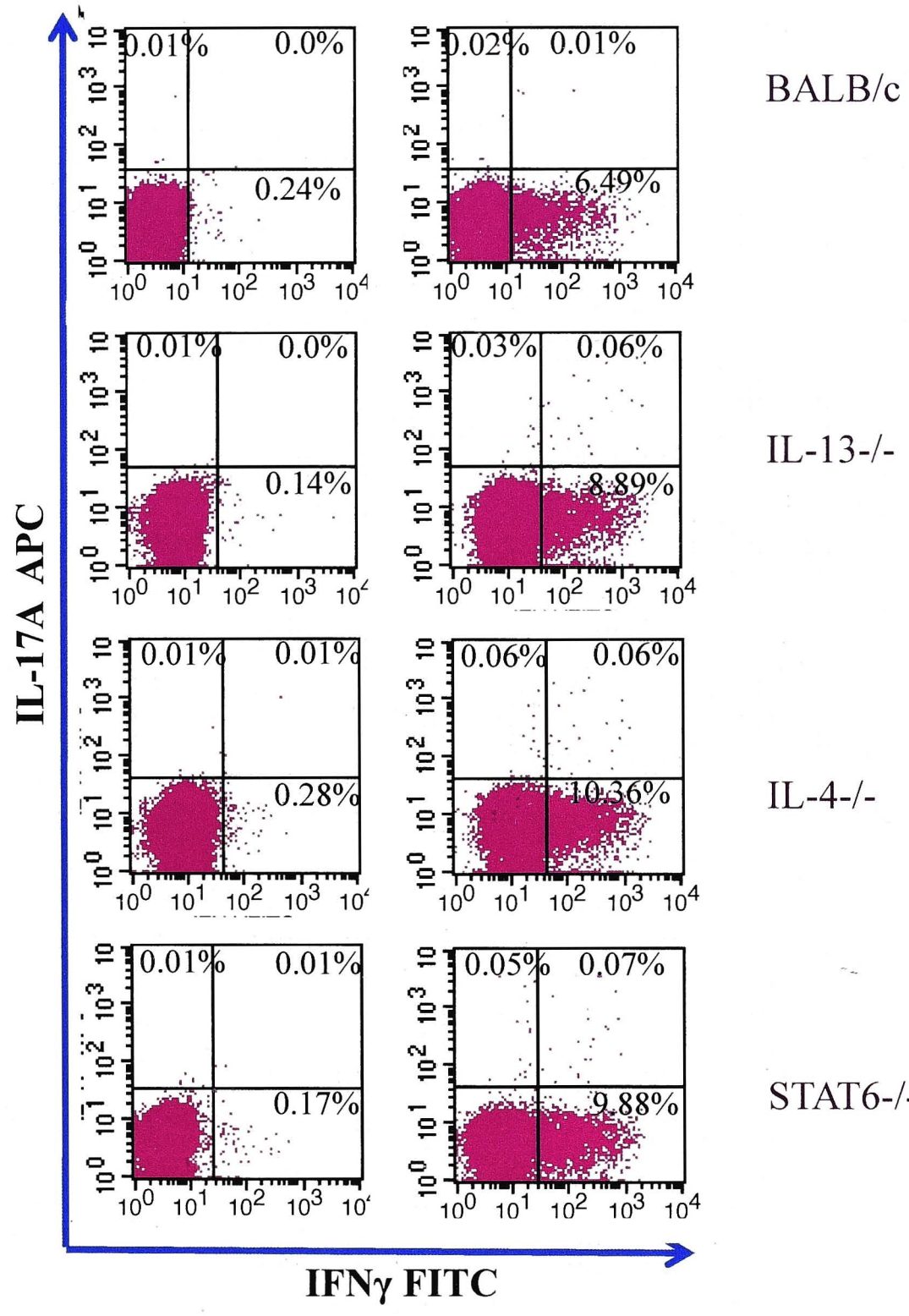

0.12
0.08
0.04
0
% of IL-17A+CD8+
BALB/c
IL-13 -/-
IL-4 -/-
STAT6 -/-

**Fig 3.9: Evaluation of lung specific IL-17A$^+$IFN-γ$^+$ (double positive) HIV-specific CD8$^+$ T cells following i.n/i.m FPV-HIV/VV-HIV prime-boost immunization**

*BALB/c, IL-13-/-, IL-4-/- and STAT6-/- (H-$^{2d}$ Background) mice (n=5) were prime-boost immunized with FPV-HIV/VV-HIV (control vaccine). At 14 days post booster immunization, lung cells were prepared as described in materials and methods. 4 x $10^6$ cells were stimulated with $K^dGag_{197-205}$ peptide for 16-20 hrs in the presence of brefeldin-A and IL-17A and IFN-γ expression by CD8$^+$ T cells was evaluated by intracellular cytokine staining. Unstimulated cells from each sample were used as background control. a) The FACS plots indicate pooled samples from each group. In all FACS plots, the upper right quadrant (R3) indicates gates R1+R2 and the numbers in the upper right quadrant indicate the percentage of CD8$^+$ T cells producing IL-17A and IFN-γ. Also, the numbers in the upper left quadrant indicate the percentage of CD8$^+$IL-17A$^+$ and the numbers in the lower right quadrant represents the percentage of CD8$^+$IFN-γ$^+$. b) The graph indicates the percentage of CD8$^+$ T cells expressing both IL-17A and IFN-γ from each group as a pooled value from n=4 mice/group. In all graphs, unstimulated cells were used as a background controls and were subtracted from each sample before plotting the data. Data are representative of two experiments.*

*(a)*


Unstimulated
Stimulated
BALB/c
IL-13-/-
IL-4-/-
STAT6-/-
IL-17A APC
IFNγ FITC
0.01%
0.0%
0.24%
0.02%
0.01%
6.49%
0.01%
0.0%
0.14%
0.03%
0.06%
8.89%
0.01%
0.01%
0.28%
0.06%
0.06%
10.36%
0.01%
0.01%
0.17%
0.05%
0.07%
9.88%

*(b)*

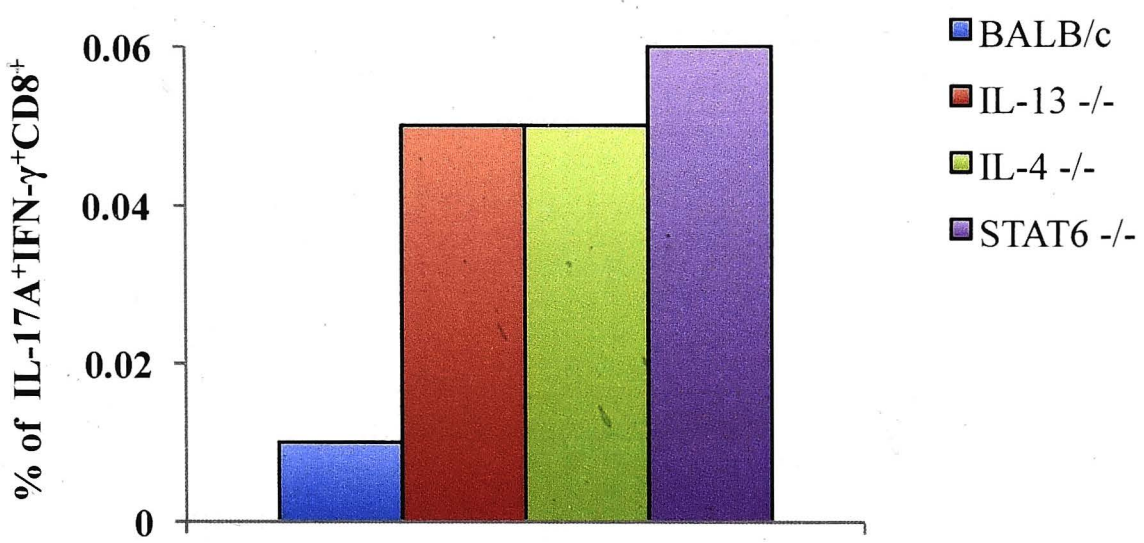
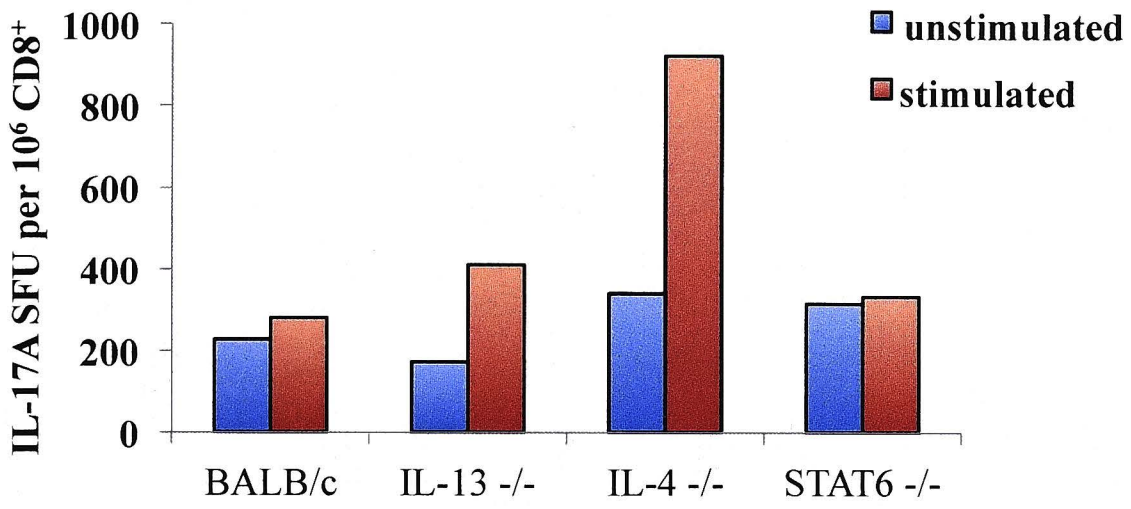

% of IL-17A+IFN-γ+CD8+
0.06
0.04
0.02
0
BALB/c
IL-13 -/-
IL-4 -/-
STAT6 -/-

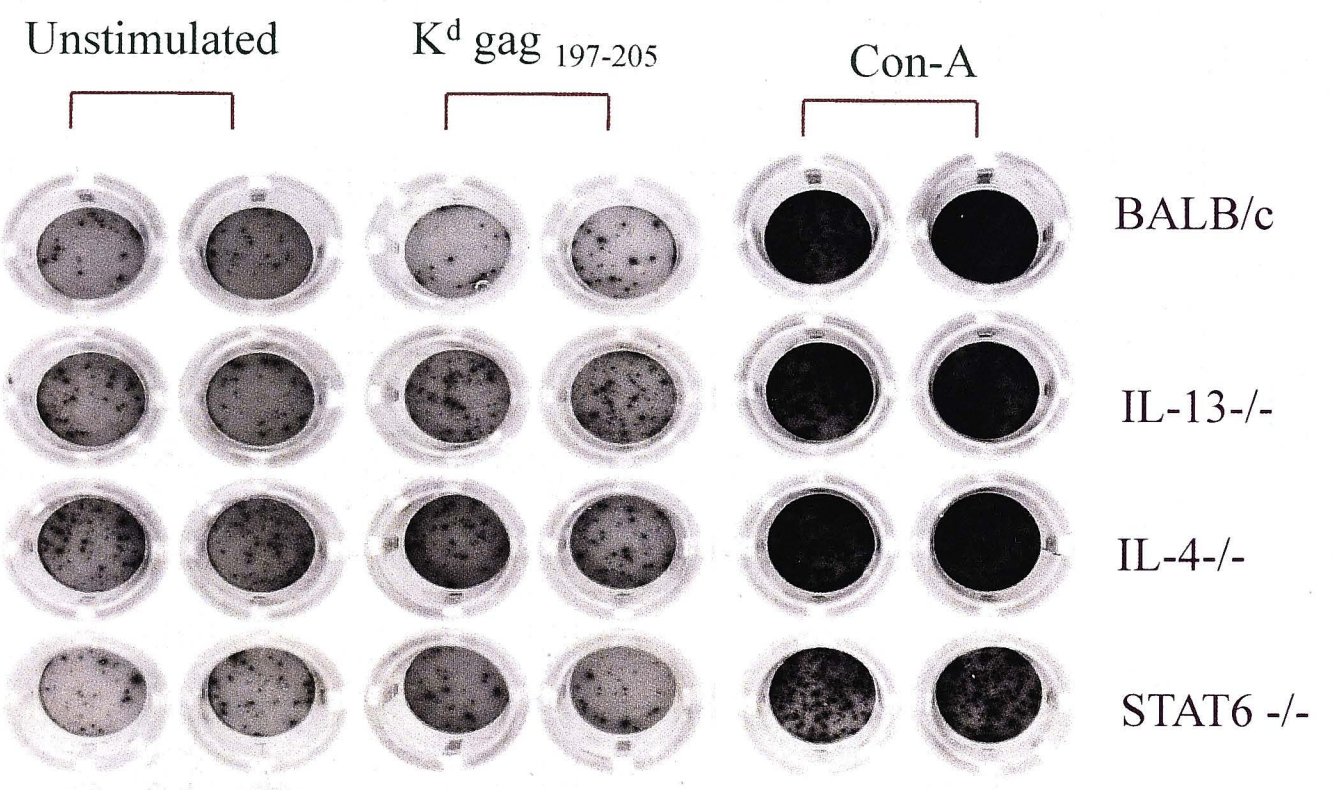
**Fig 3.10: Determining IL-17 expressions in the lung using ELIspot**

*Mice (n=4) from BALB/c, IL-13-/-, IL-4-/- and STAT6-/- (H-$^{2d}$ Background) were prime-boost immunized with FPV-HIV/VV-HIV. At 14 days post booster immunization, lung samples were prepared and IL-17A expression was measured by a) ELIspot, c) Intracellular cytokine staining as described previously. Unstimulated cells from each sample were used as background control. a) The graph indicates the total number IL-17A SFU compared to the number of IL-17A cells per $10^6$ $CD8^+$ T cells measured by ICS. b) Photograph of lung ELIspot plate. Data are representative of two experiments.*

***(a) ELIspot***




***(b)***




***(c) ) ICS as $10^6$ CD8 T cells***

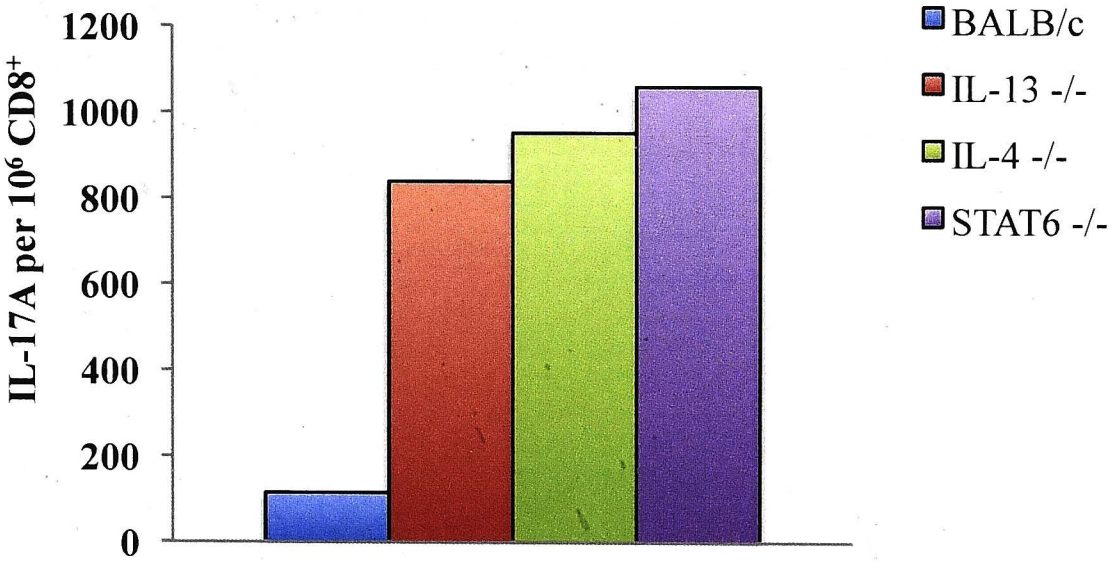
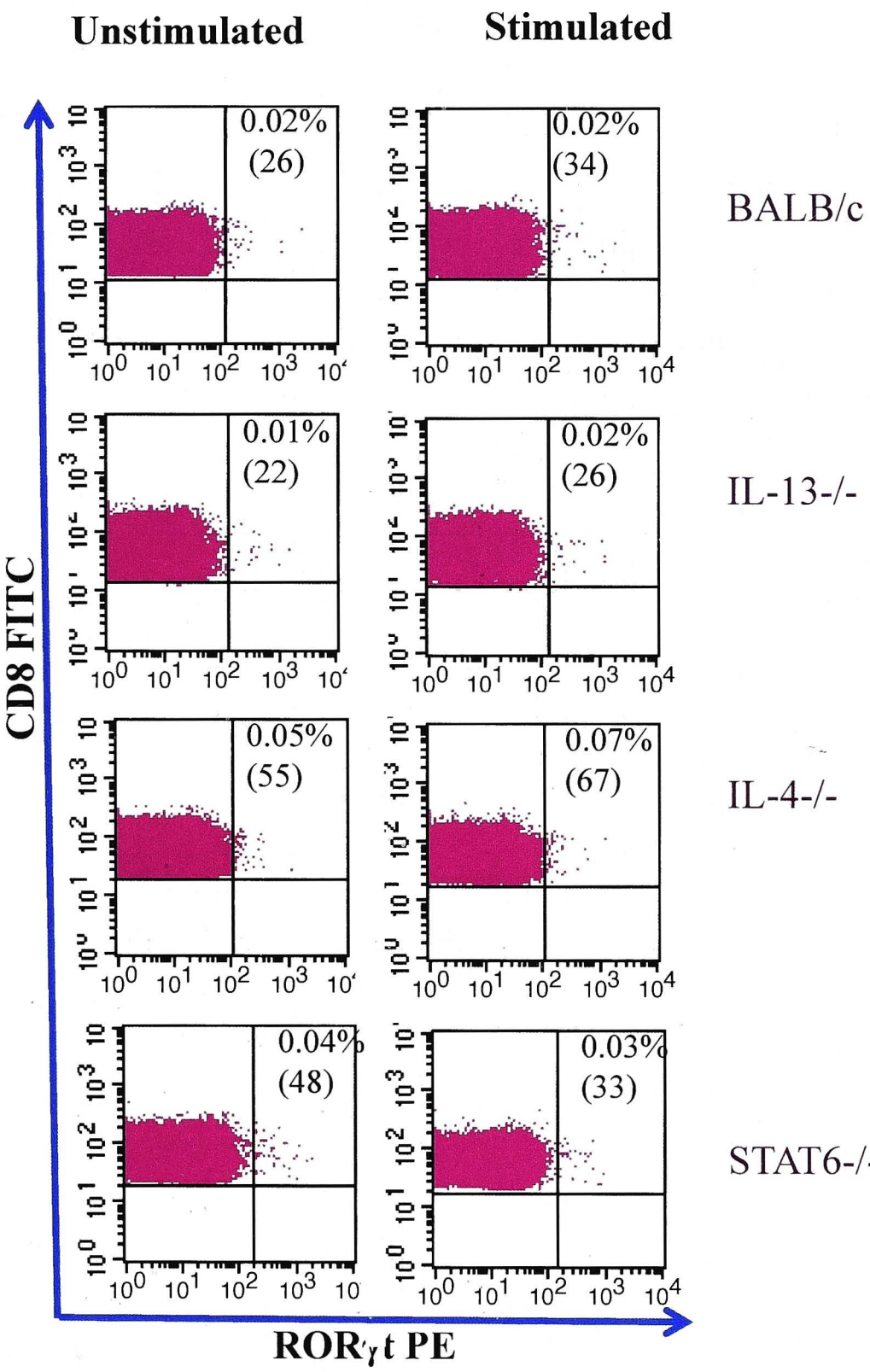


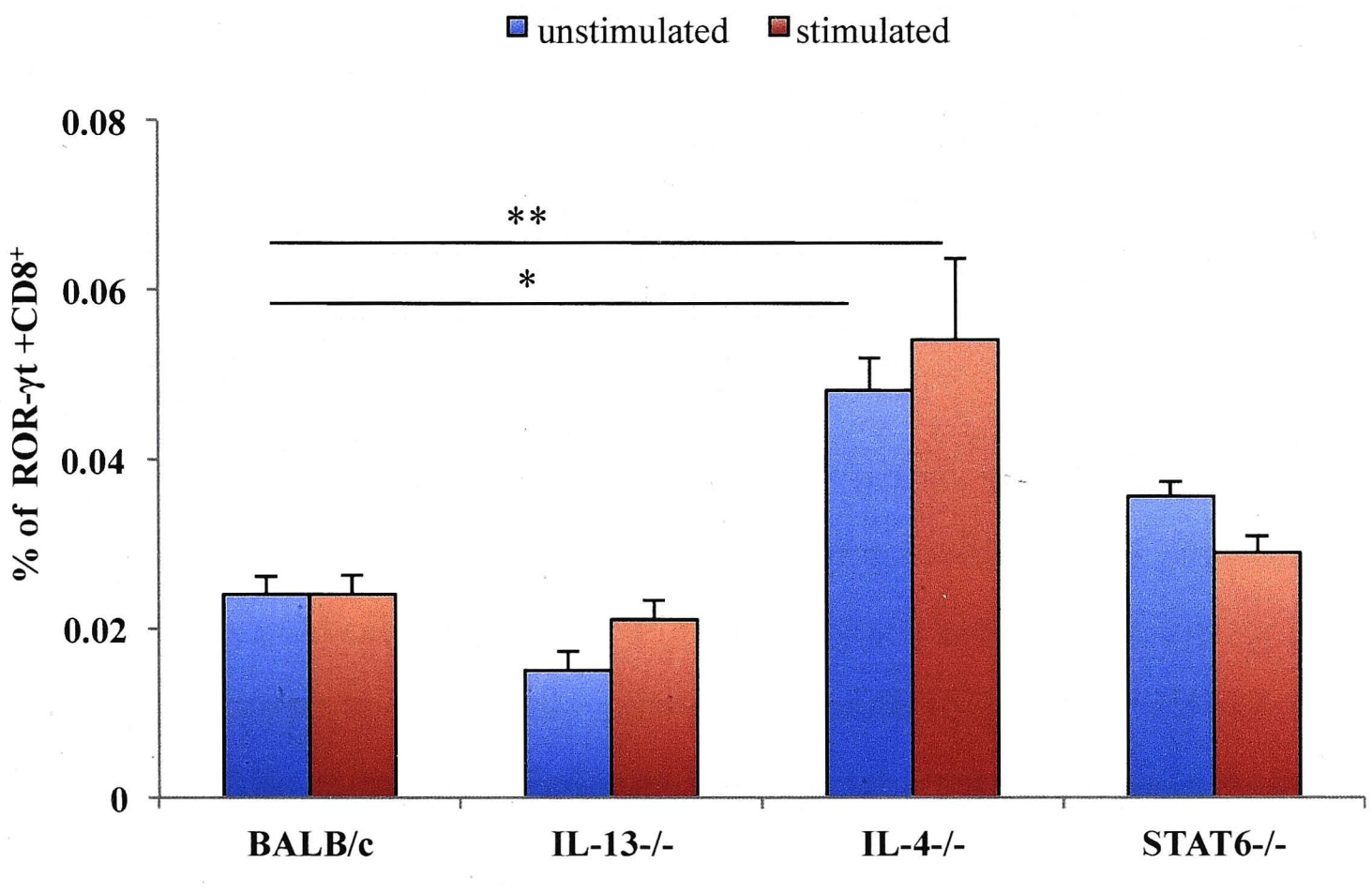
**Fig 3.11: Evaluation of ROR-γt expression following i.n/i.m FPV-HIV/VV-HIV prime-boost immunization**

*Mice (n=6) from BALB/c, IL-13-/-, IL-4-/- and STAT6-/- (H-$^{2d}$ Background) were prime-boost immunized with FPV-HIV/VV-HIV. At 14 days post booster immunization, 4 x $10^6$ cells were stimulated with $K^dGag_{197-205}$ peptide for 6 hrs and the expression of ROR-γt by $CD8^+$T cells were evaluated by intracellular cytokine staining. Unstimulated cells from each sample were used as background control. a) The FACS plots indicate representative animals from each group. In all FACS plots, the upper right quadrant (R3) indicate gates R1+R2 and the numbers indicate the percentage of $CD8^+$ T cells producing ROR-γt (top) and also the gated number of events (bottom) within brackets. b) Graph indicates the percentage of $CD8^+$ T cells expressing ROR-γt from n=6 mice /group. The error bars represent standard error of the mean (SEM). p values were calculated using student's t test *p=0.02, (BALB/c Vs. IL-4-/- Unstimulated), **p= 0.01(BALB/c Vs. IL-4-/- stimulated).*

*(a)*


Unstimulated
Stimulated
0.02%
(26)
0.02%
(34)
BALB/c
0.01%
(22)
0.02%
(26)
IL-13-/-
0.05%
(55)
0.07%
(67)
IL-4-/-
0.04%
(48)
0.03%
(33)
STAT6-/-
CD8 FITC
RORγt PE

***(b)***


unstimulated
stimulated
0.08
0.06
0.04
0.02
0
% of ROR-γt +CD8+
**
*
BALB/c
IL-13-/-
IL-4-/-
STAT6-/-

### 3.7 Discussion

In the current study, both IL-17A ELIspot and ICS data, clearly indicated that the expression of IL-17A by HIV specific $CD8^+$ T cells is significantly enhanced in IL-13-/-, IL-4-/- and STAT6-/- mice compared to wild type BALB/c suggesting that these cytokines were involved in modulating IL-17A. Out of the three KO mice groups tested, IL-4-/- mice showed the highest IL-17A expression in both the spleen and the lung. It is well established that IL-4 and IL-13 are closely related and are regulated in a STAT6 dependent manner [152, 173]. Current observations were also consistent with some of the recent findings where the absence of IL-4 and IL-13 during epicutaneous sensitization was shown to enhance IL-17 production in IL-4 and IL-13 KO mice in both systemic compartment and lung. Also, among IL-4-/-, IL-13-/- and IL-4/IL-13 DKO mice, IL-4 was identified as the key cytokine in down-regulating IL-17 expression in an asthma model [69]. Conney *et al.* have shown that IL-4 through STAT6 regulate STAT3 inhibition of IL-17 at the IL-17A promoter [174]. Since IL-13 and IL-4 share a common STAT6 cell signaling regulatory pathway via IL-4Rα and IL-13Rα1, there is a great possibility that both these cytokines regulate the expression of IL-17A. The results obtained with STAT6-/- mice further substantiate this hypothesis.

Studies by Cruz *et al.* have shown that following *Mycobacterium bovis bacille Calmette Guérin* infection, IFN-γ deficient mice produced elevated number of IL-17-producing T cells [68]. Also, addition of exogenous IFN-γ increased IL-12 and decreased IL-23 mediated $CD4^+$IL-17A expression suggesting that IFN-γ down regulate IL-17A expression. However, current data clearly demonstrate that in our HIV prime-boost model, high IFN-γ expression does not correlate with the regulation of IL-17A expression in $CD8^+$ T cells. Furthermore, the majority of HIV-specific IL-17A expressing CTLs also expressed IFN-γ in both the spleen and lung. Also, the highest $CD8^+$IL-17A$^+$IFN-γ$^+$ was observed in IL-4 KO mice. Consistent with our findings, Weaver *et al.* have shown the emergence of IFN-γ producing CD4 cells from Th17 precursors in an IL-12-driven manner [175]. Studies have shown that these IL-17-secreting cells can convert into IFN-γ secreting cells suggesting that the cell phenotypes that express IL-17A may be functionally plastic [176]. Moreover, these studies indicated that the transcription factor STAT3 could be involved in the polarization of IL-17 expressing cells and play a functional role in generating the IL-17A$^+$IFN-γ$^+$CD8 T cells [176].

Various studies have indicated that ROR-γt is one of the transcription factors involved in the regulation of IL-17A producing cells [59, 65, 172, 177]. As transcription factors normally get activated several times prior to the protein expression, the expressions of ROR-γt in HIV-specific $CD8^+$ T cells were analyzed at 6 hrs following HIV-specific peptide stimulation. Although, results showed no significant difference in the ROR-γt protein expression in HIV-specific $CD8^+$ T cells between unstimulated and stimulated cells, there was an overall increased production of ROR-γt in IL-4-/- and STAT6-/- mice compared to wild type control mice. The ICS data have shown that IL-4-/- and STAT6-/- mice produced higher IL-17A upon $K^dGag_{197\text{-}205}$ peptide stimulation. This clearly suggests that ROR-γt plays an important role in regulating IL-17A expression by HIV specific $CD8^+$ T cells in IL-4-/- and STAT6-/- mice. Newcomb *et al.* have demonstrated that Th2 cytokines down regulate the ROR-γt expression either at mRNA level or under Th17 polarizing conditions [71]. Although IL-13-/- mice produced elevated IL-17A expression, ROR-γt expressions were similar to BALB/c mice suggesting that in IL-13-/- mice, IL-17A expression may be regulated in a different manner or a time frame compared to IL-4 KO mice. Therefore, the expressions of other IL-17A regulating factors at the mRNA level were also evaluated in IL-4 and IL-13 KO mice, which form the basis of the project (chapter 4).

Upon MHC-I peptide stimulation, different cytokines are expressed at different time intervals and are regulated in a different manner in HIV-specific $CD8^+$ T cells. For example, Ranasinghe *et al.* have shown that IL-2 and TNF-α were expressed by HIV-specific $CD8^+$ T cells at 3-4 hrs following $K^dGag_{197\text{-}205}$ peptide stimulation whereas IFN-γ expression can peak from 6-16 hrs [144]. Similarly, when the expression kinetics of IL-17A was evaluated at both 6 hrs and 16 hrs of $K^dGag_{197\text{-}205}$ peptide stimulation no significant difference in the protein expression was observed suggesting that the expression of IL-17A is more similar to the kinetics of IFN-γ.

In conclusion, the data in this chapter clearly indicate that IL-4 and IL-13 can modulate IL-17A expression by HIV-specific $CD8^+$ T cells in systemic (spleen) and mucosal (lung) compartments. In contrast, IFN-γ is not involved in the regulation of IL-17A in HIV specific $CD8^+$ T cells, as most of the IL-17A producing $CD8^+$ T cells were also positive for IFN-γ. Previously, Ranasinghe *et al.* have shown that IL-4, IL-13 and STAT6 are involved in regulating the avidity of HIV-specific $CD8^+$ T cells [165] and

protective immunity Ranasinghe *et al.* (Submitted June 2012). In this study, IL-4, IL-13 and STAT6 have also been shown to modulate IL-17A expression by HIV-specific $CD8^+$ T cells; data suggest that IL-17A could play a direct or indirect role in modulating HIV-specific $CD8^+$ T cell avidity.

# CHAPTER 4: Regulation of IL-17 A in HIV-specific $CD8^+$ T cells following HIV-1 prime-boost immunization

## 4.1 Introduction

The differentiation of T cell subsets into Th1 or Th2 or Th17 cells is regulated by the lineage specific cytokines which signals through their STAT proteins to activate their corresponding transcription factors [178]. These transcription factors can concurrently promote their own lineage differentiation and inhibit its alternative-differentiating pathway (Gene repression) [179]. For example, the expression of i) T-bet differentiates Th1 pathway, ii) GATA3 mediate Th2 pathway, iii) ROR-γt in Th17 pathway and iv) Foxp3 in Treg differentiation (Fig 4.1).

Th17 cells have emerged as an independent subset of T cells and exhibit distinct cytokine and transcriptional profile [50]. The differentiation of Th17 cells begins with the combined initiation of IL-6 and TGF-β to activate the Janus-kinase pathway thereby initiating STAT3 signaling of ROR-γt transcription [180]. Following differentiation, IL-21 is required for the amplification of Th17 response and IL-23 is involved in the maintenance of Th17 expression (Fig 4.2).

Results from previous chapter (3) have shown that IL-4, IL-13 and STAT6 regulate the expression of IL-17A following HIV prime-boost immunization. Therefore, in this study, the expression IL-17A regulatory functions in $CD8^+$ T cells were evaluated following HIV-specific ($K^dGag_{197-205}$) peptide stimulation in BALB/c, IL-4-/-, IL-13-/- and STAT6-/- mice at the mRNA level. Specifically, the expression of cytokines (IFN-γ, IL-6, IL-17A, IL-23a), and transcriptional factors (T-Bet, TGF-β, GATA3, and ROR-γt) in $K^dGag_{197-205}$ specific $CD8^+$ T cells were measured. In addition, relative expressions of T-bet and GATA3 were also evaluated to elucidate whether these played any role in IL-17A regulation. Finally, the expression of above gene functions were also examined in $K^dGag_{197-205}$ tetramer reactive $CD8^+$ T cells, (where the cells were not stimulated) at 500-sorted cells per group.

## 4.2 Understanding the transcriptional regulation profile of IL-17A in BALB/c and GKO mice:

The transcriptional regulation of IL-17A following i.n/i.m. FPV-HIV/VV-HIV prime-boost immunization was characterized using IL-13-/-, IL-4-/-, STAT6-/- and BALB/c control mice. $CD8^+$ T cells were negatively isolated and were stimulated with $K^dGag_{197-}$

$_{205}$ peptide for 16 hrs. RNA was extracted, cDNA was synthesized and RT-PCR was performed.

Firstly, the mRNA expressions of TGF-β, IL-6 were analyzed and data indicated that the relative expressions of these factors were highly elevated in IL-4-/- mice (Fig 4.3). The expression levels were not significantly different in BALB/c, IL-13-/- and STAT6-/- mice. When the ROR-γt mRNA level was examined, elevated mRNA copy numbers were observed in IL-4-/-, IL-13-/- and STAT6-/- mice compared to BALB/c mice. However, no elevated expression of IL-23a mRNA by HIV-specific $CD8^+$ T cells were observed in all the mice tested. Finally, when the IL-17A mRNA levels were compared against the transcriptional factors, IL-17A mRNA profile correlated with TGF-β and ROR-γt expressions eliciting higher levels in IL-4-/-, IL-13-/-, STAT6-/- compared to BALB/c control mice (Fig 4.3). In summary, current data suggests that the transcriptional regulation of IL-17A in HIV-specific $CD8^+$ T cells was dependent on IL-4, IL-13 and STAT6. More strikingly, TGF-β, IL-6, ROR-γt and IL-17A mRNA played a significant role in IL-4-/- mice compared to IL-13-/- or STAT6-/- mice (Table 4.1).

As at 16 hrs post HIV-specific peptide stimulation, IL-6 and IL-23a mRNA levels were found to be low in these HIV-specific $CD8^+$ T cells. Therefore, the expression kinetics of these IL-17A regulatory factors were also examined at 4 hrs and compared with 16 hrs. Interestingly, data indicated no significant elevated expression of IL-17A, TGF-β, IL-6, ROR-γt or IL-23a was observed at 4 hrs of peptide stimulation compared to 16 hrs (Fig 4.4).

### 4.3 Role of T-bet and GATA3 expression in IL-17A regulation:

Transcription factors T-bet (regulating IFN-γ) and GATA3 (down stream regulator of IL-4/IL-13) mRNA expressions levels were also assessed to confirm whether the expression of these transcription factors played any role in IL-17A regulation in HIV-specific $CD8^+$ T cells (Fig 4.5). Although the IL-17A mRNA expression profile was IL-4-/- > IL-13-/- > STAT6-/- > BALB/c, this pattern did not correlate with T-bet and GATA3 mRNA expressions profiles (Fig 4.6). For example, BALB/c mice that expressed T-bet and GATA3 mRNA did not produce IL-17A or ROR-γt (Fig 4.3). This clearly indicated that T-bet and GATA3 were not involved in the regulation of IL-17A in HIV-specific $CD8^+$ T cells. Interestingly, T-bet mRNA levels were also elevated in

**Fig 4.1: Schematic diagram of differentiation of Th1, Th2, Th17 and Treg cells**

*T cells are divided into at least four lineages, namely, Th1, Th2, Th17 and Treg. These cells express master regulatory transcription factors T-bet, GATA3, ROR-γt and Foxp3 that regulate IFN-γ, IL-4, IL-17 and TGF-β, IL-10 expression respectively.*

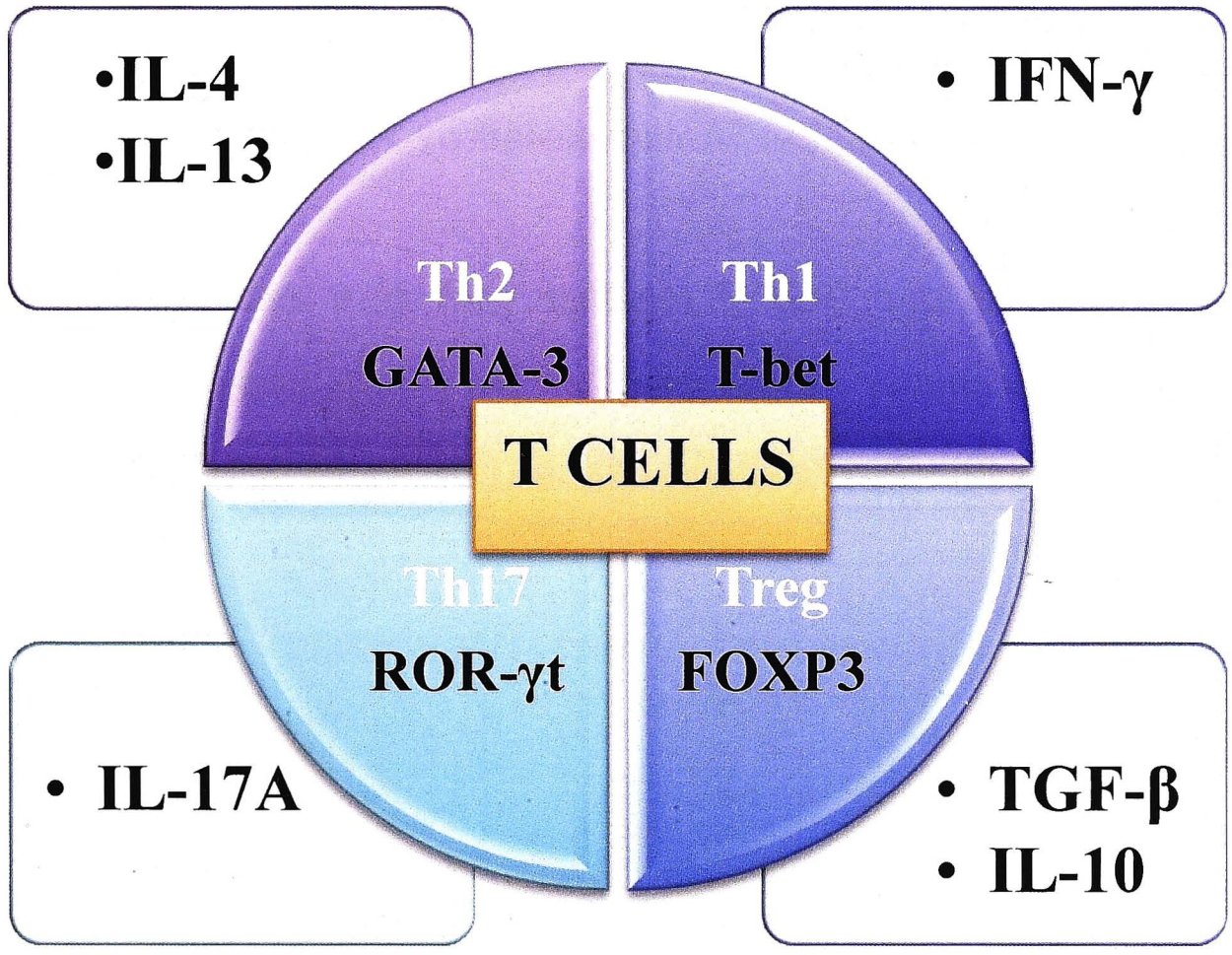

•IL-4
•IL-13
• IFN-γ
Th2
GATA-3
Th1
T-bet
T CELLS
Th17
ROR-γt
Treg
FOXP3
• IL-17A
• TGF-β
• IL-10

**Fig 4.2: Regulation of IL-17A expression by Th17 cells**

*It is established that TGF-β together with IL-6 enhances expression of ROR-γt and promotes development of Th17 cells secreting IL-17A. Once differentiated, IL-23 stabilizes the IL-17A expression through ROR-γt signaling (180).*

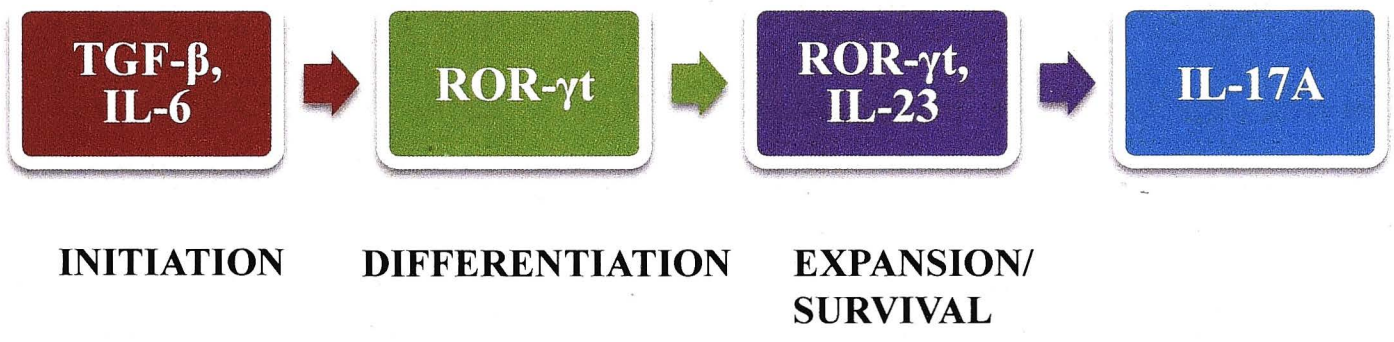

TGF-β, IL-6
ROR-γt
ROR-γt, IL-23
IL-17A
INITIATION
DIFFERENTIATION
EXPANSION/ SURVIVAL

**Fig 4.3: Transcriptional regulation profile of IL-17A in BALB/c and KO mice in HIV-specific $CD8^+$ T cells following i.n/i.m prime-boost immunization**

*BALB/c, IL-13-/-, IL-4-/- and STAT6-/- (H-$^{2d}$ Background) mice (n=4) were prime-boost immunized with FPV-HIV/VV-HIV (control vaccine). At 2 weeks post booster immunization, $CD8^+$T cells from splenocytes were negatively enriched and stimulated with $K^dGag_{197-205}$ peptide for 16 hrs. RNA was isolated, cDNA was synthesized and real-time PCR was performed in duplicates to assess the changes in the mRNA levels. Data show the relative mRNA copy numbers of TGF-β, IL-6, ROR-γt, IL-23a and IL-17A of stimulated cells after normalizing against L32 expression. These experiments were repeated twice and similar trends were observed.*

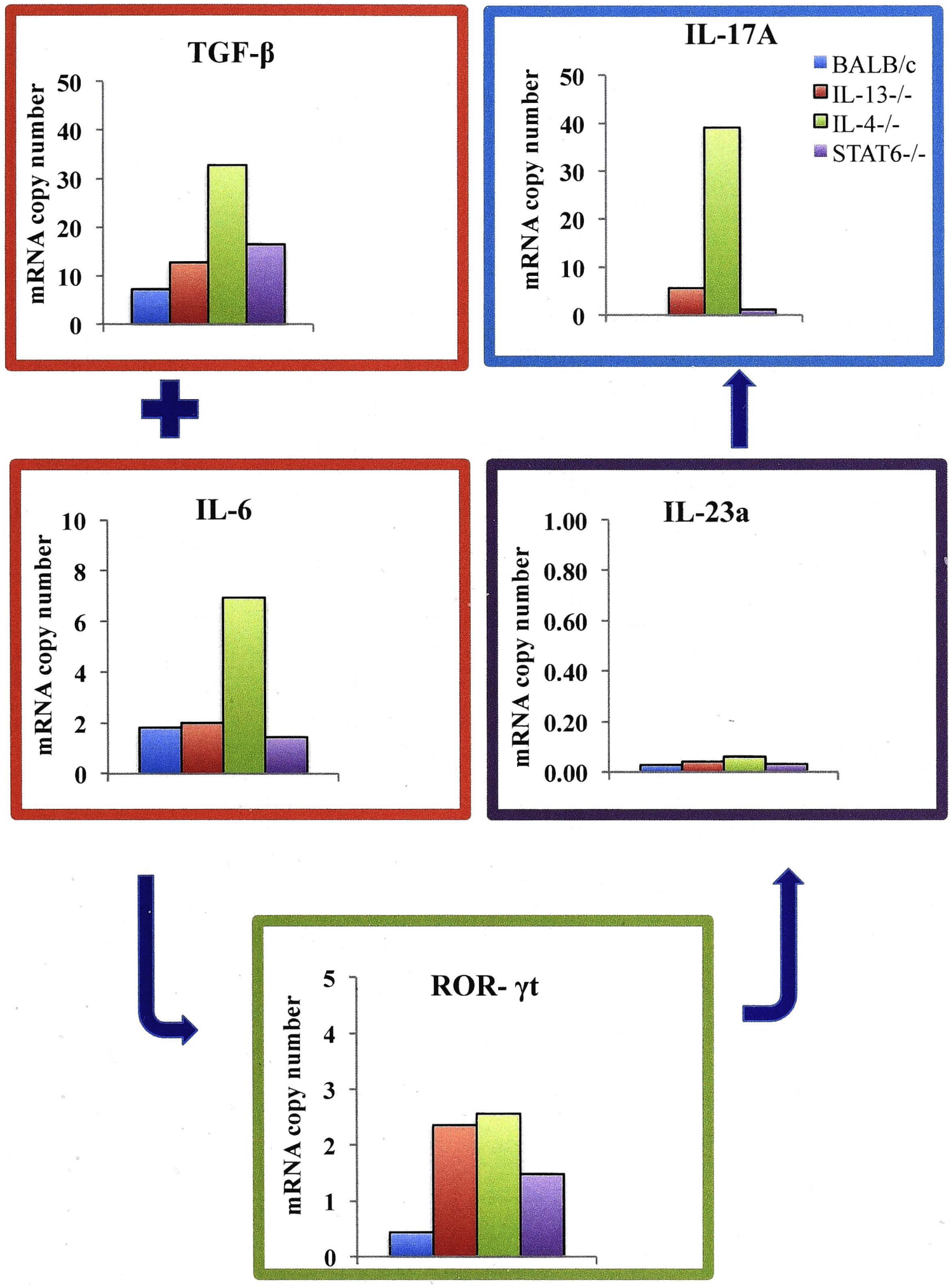

TGF-β
mRNA copy number
50
40
30
20
10
0
IL-17A
BALB/c
IL-13-/-
IL-4-/-
STAT6-/-
IL-6
10
8
6
4
2
IL-23a
1.00
0.80
0.60
0.40
0.20
0.00
ROR- γt
5
4
3
1

**Table 4.1: Summary of IL-17A regulation following prime-boost immunization**

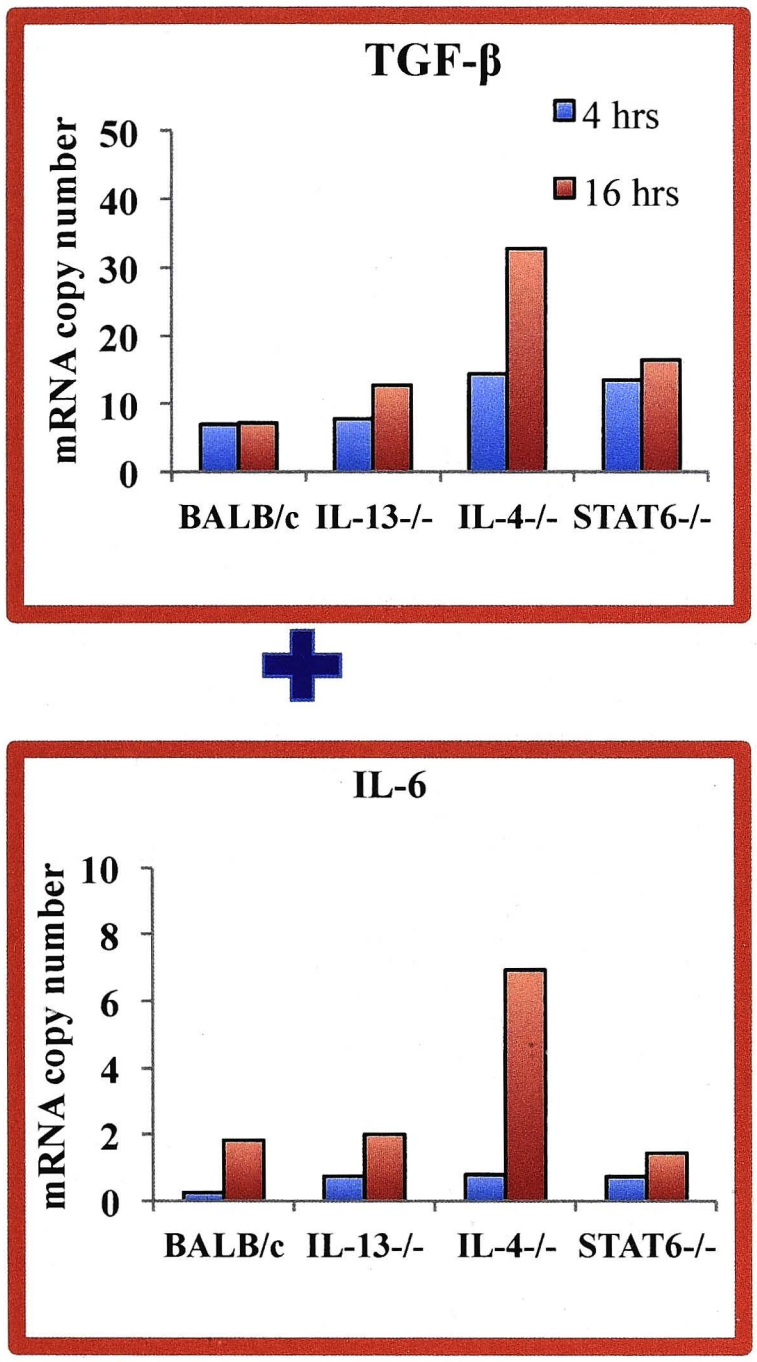
*Data indicate that the mRNA copy numbers of IL-6, IL-17A, IL-23a, TGF-β and ROR-γt were elevated in IL-4-/- mice compared to the other groups tested.*

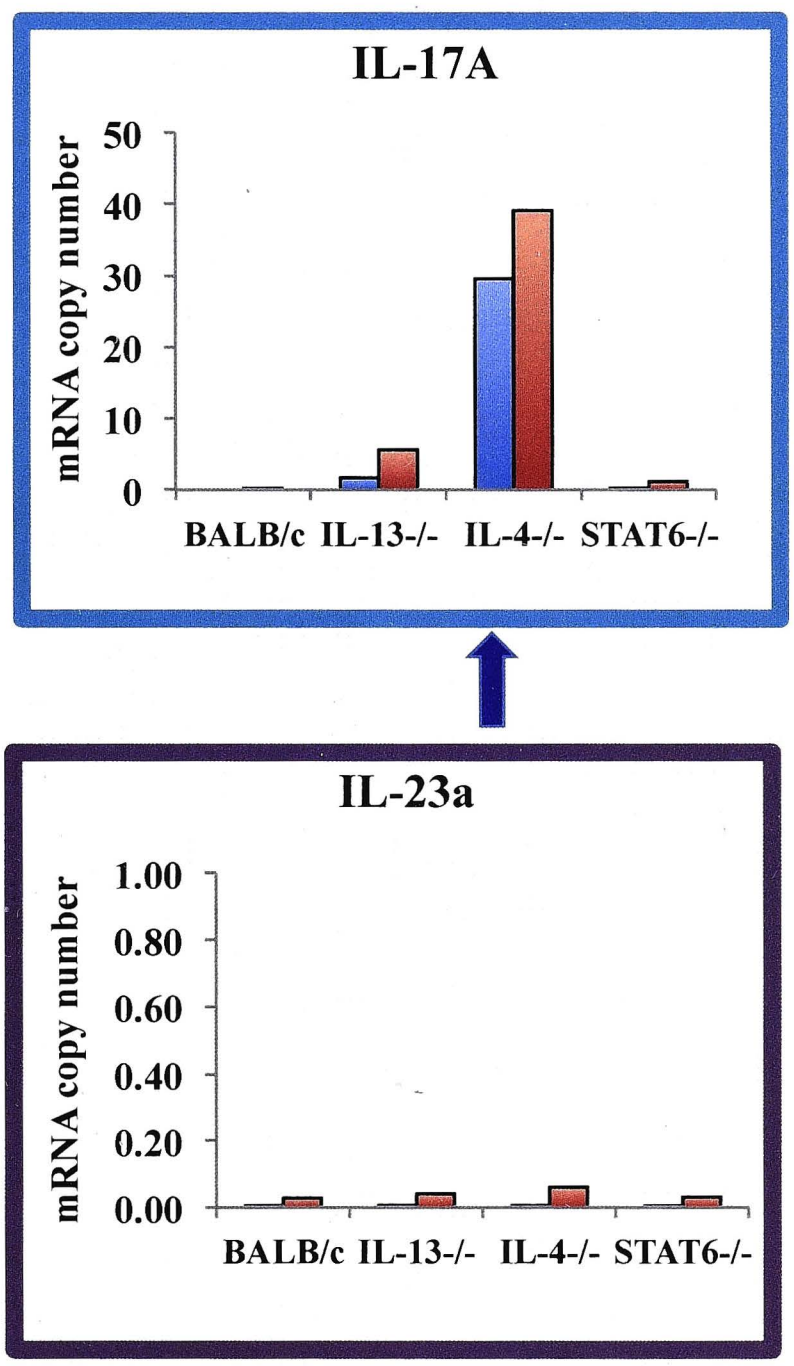
**Note: No expression of IL-23a was detected.*

| | IL-6 | TGF-β | ROR-γt | IL-23 * | IL-17 |
|---|---|---|---|---|---|
| IL-4-/- | +++ | +++ | ++± | − | ++++ |
| IL-13-/- | + | ++ | ++± | − | ++ |
| STAT6-/- | ± | ++ | +± | − | + |
| BALB/c | + | ± | ± | − | - |

**Fig 4.4: Expression kinetics of IL-17A regulating factors in HIV-specific $CD8^+$ T cells following prime-boost immunization**

*BALB/c, IL-13-/-, IL-4-/- and STAT6-/- (H-$^{2d}$ Background) mice (n=4) were prime-boost immunized with FPV-HIV/VV-HIV (control vaccine). At 2 weeks post-booster immunization, $CD8^+$T cells from splenocytes were negatively enriched and stimulated with $K^dGag_{197-205}$ peptide for 4 hrs and 16 hrs. RNA was isolated at different time points, cDNA was synthesized and real-time PCR was performed in duplicates to assess the changes in the mRNA levels at these different time-points. Data show TGF-β, IL-6, ROR-γt, IL-23a and IL-17A mRNA copy numbers at 4 hrs and 16 hrs of $K^dGag_{197-205}$ peptide stimulation normalized against the house-keeping L32 mRNA expression. These experiments were repeated twice and similar trends were observed.*


TGF-β
4 hrs
16 hrs
mRNA copy number
50
40
30
20
10
0
BALB/c IL-13-/- IL-4-/- STAT6-/-


IL-17A
mRNA copy number
50
40
30
20
10
0
BALB/c IL-13-/- IL-4-/- STAT6-/-


IL-6
mRNA copy number
10
8
6
4
2
0
BALB/c IL-13-/- IL-4-/- STAT6-/-


IL-23a
mRNA copy number
1.00
0.80
0.60
0.40
0.20
0.00
BALB/c IL-13-/- IL-4-/- STAT6-/-

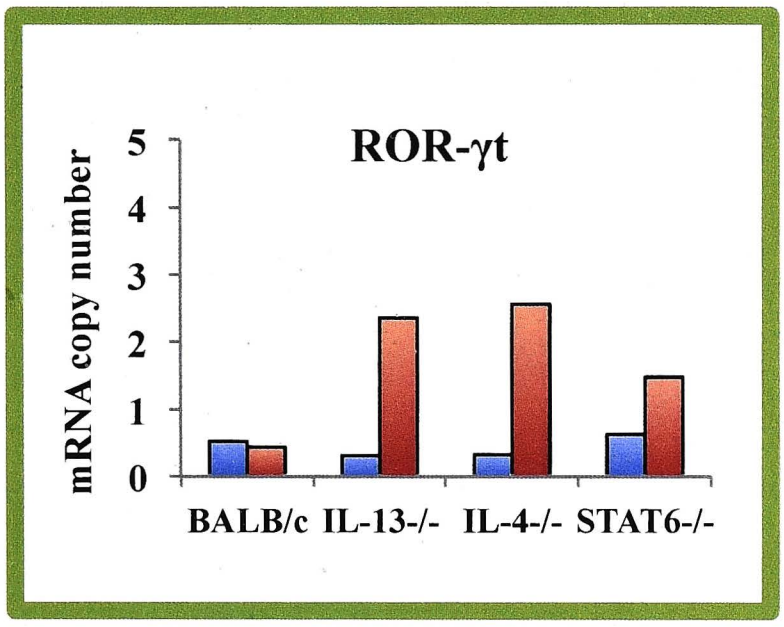

ROR-γt
mRNA copy number
5
4
3
2
1
0
BALB/c IL-13-/- IL-4-/- STAT6-/-

**Fig 4.5: Schematic diagram indicating the regulation of IL-17A in HIV-specific $CD8^+$ T cells**

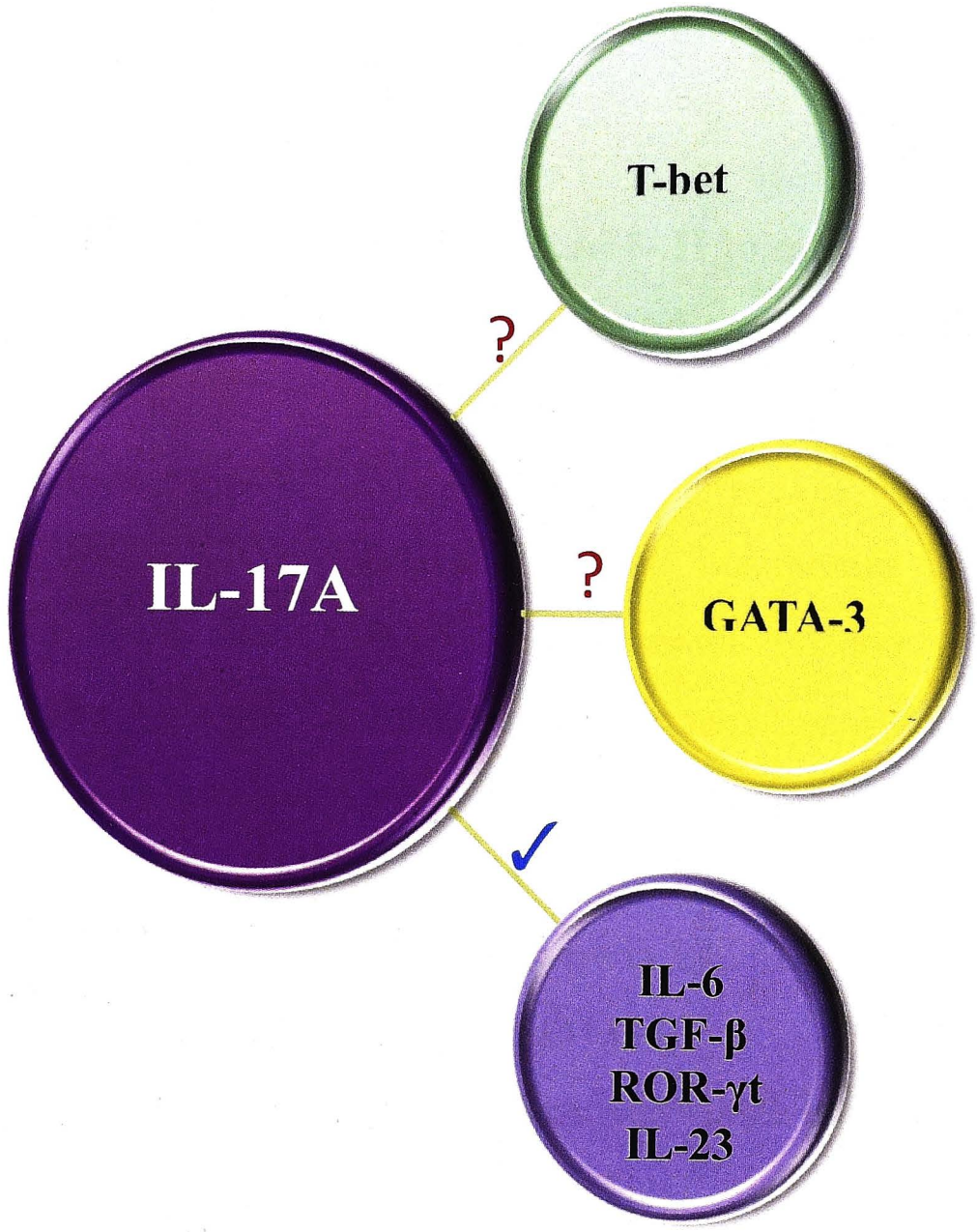
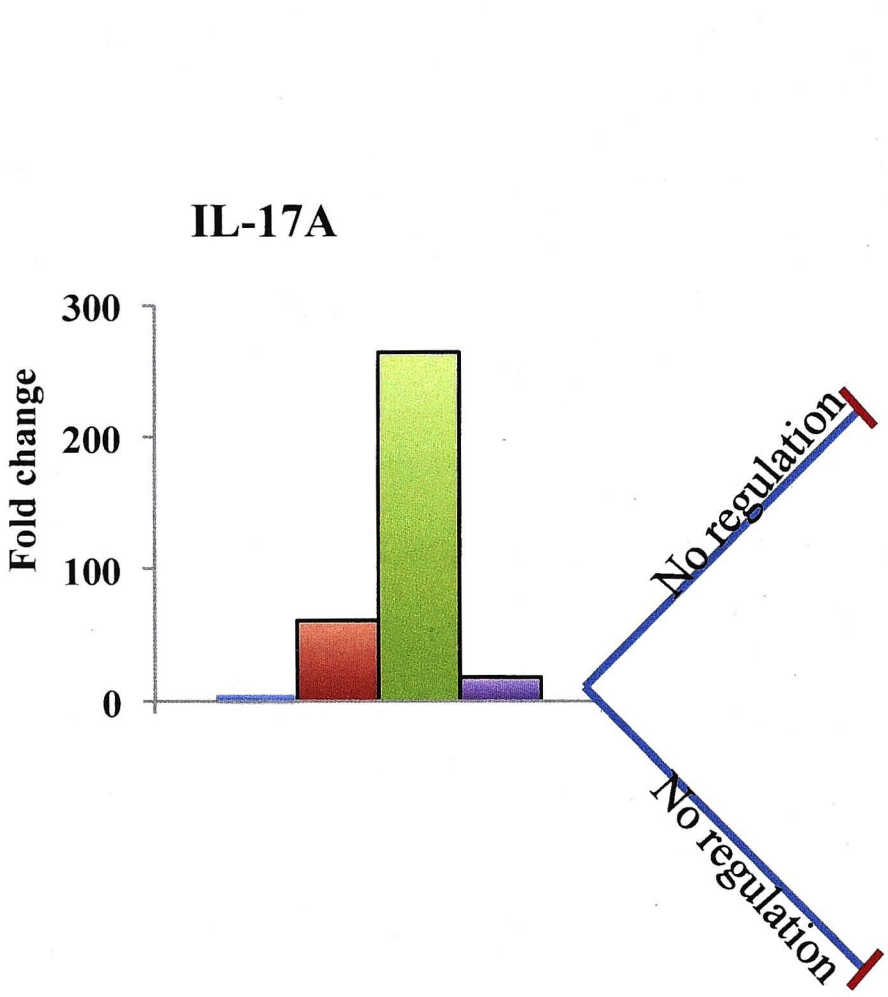
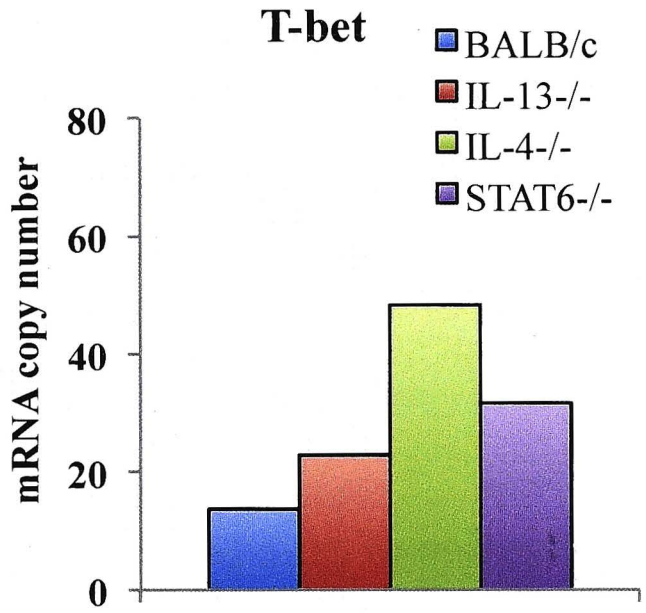
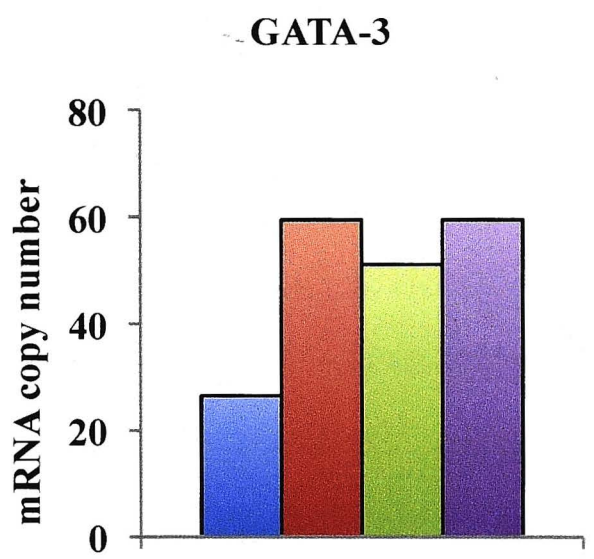

T-bet
?
IL-17A
?
GATA-3
✓
IL-6
TGF-β
ROR-γt
IL-23

**Fig 4.6: Evaluation of IL-17A, T-bet and GATA3 in HIV-specific $CD8^+$ T cells following prime-boost immunization in BALB/c and KO mice**

*BALB/c, IL-13-/-, IL-4-/- and STAT6-/- (H-$^{2d}$ Background) mice (n=4) were prime-boost immunized with FPV-HIV/VV-HIV (control vaccine). At 2 weeks post booster immunization, $CD8^+$ T cells from splenocytes were negatively enriched and stimulated with $K^dGag_{197-205}$ peptide for 16 hrs. RNA was isolated, cDNA was synthesized and real-time PCR was performed to assess the changes in the mRNA profiles of the transcription factors T-bet and GATA3 compared to IL-17A. Graph show that the mRNA expression of IL-17A, T-bet, GATA3 normalized against L32 expression. These experiments were repeated twice and similar trends were observed.*


IL-17A
300
200
100
0
Fold change
No regulation
No regulation


T-bet
BALB/c
IL-13-/-
IL-4-/-
STAT6-/-
80
60
40
20
0
mRNA copy number


GATA-3
80
60
40
20
0
mRNA copy number

IL-4-/- mice compared to other KO mice, which correlated with the IFN-γ production by these mice (Fig 4.7).

### 4.4 Evaluation of IFN-γ and IL-17A mRNA expression following $K^dGag_{197-205}$ peptide stimulation:

As some reports have suggested that IFN-γ negatively regulate IL-17A expression, in this study the expression of IFN-γ and IL-17A mRNAs were again assessed to see whether IFN-γ influenced IL-17A expression at the transcriptional level. As the expression of both IFN-γ and IL-17A mRNA (Fig 4.7), and protein (Fig 3.2) profile were very similar in HIV-specific $CD8^+$ T cells eliciting (IL-4-/- > IL-13-/- > STAT6-/- > BALB/c) (Fig 4.7), data clearly indicated that IFN-γ did not modulate the expression of IL-17A even at the transcription level or translational level. The T-bet expression pattern further substantiated these observations (4.6).

### 4.5 Evaluation of granzyme-B and IL-17A mRNA expression in HIV-specific $CD8^+$ T cells following prime-boost immunization:

Studies have shown that $CD8^+$ T cells producing IL-17 can be less cytotoxic [181], therefore, the expressions of IL-17A and granzyme-B were also assessed following $K^dGag_{197-205}$ peptide stimulation as discussed in materials and methods. Results showed that the relative mRNA expression of IL-17A and granzyme-B were higher in KO mice compared to wild type BALB/c mice (Fig 4.8). The mRNA expression profile of granzyme-B was IL-13-/- > STAT6-/- ≥ IL-4-/- > BALB/c. Interestingly, the IL-4-/- and IL-13-/- granzyme-B expression inversely correlated with the IL-17A expression in these mice. This data is consistent with previous findings in our laboratory where elevated granzyme-B by IL-13-/- HIV-specific $CD8^+$ T cells were observed compared to BALB/c mice [165].

### 4.6 Evaluation of cytokines and transcription factors in HIV-tetramer reactive $CD8^+$ T cells:

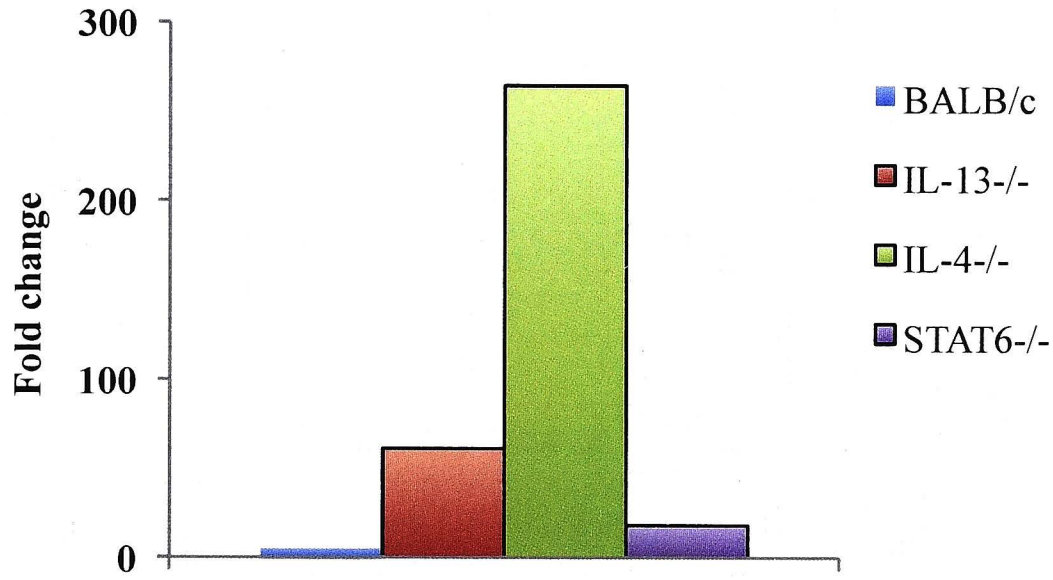
The transcription factors that were involved in IL-17A regulation were also analyzed in HIV-specific tetramer reactive $CD8^+$ T cells. Following prime-boost immunization, tetramer specific $CD8^+$ T (500 cells/well) were FACS sorted into 96 well plates, cDNA was synthesized (without any $K^dGag_{197-205}$ peptide stimulation) and RT-PCR was

performed. No IL-6, IL-17A, TGF-β and ROR-γt mRNA was detected in these tetramer specific $CD8^+$ T cells unlike IFN-γ and granzyme-B expression as observed in previous studies [144]. This indicated that IL-17A was not induced when cells were not stimulated with HIV-specific peptide.

**Fig 4.7: Evaluation of IL-17A and IFN-γ mRNA expression in HIV-specific $CD8^+$ T cells from BALB/c, IL-4-/-, IL-13-/-, and STAT6-/- mice following prime-boost immunization**

*BALB/c, IL-13-/-, IL-4-/- and STAT6-/- ($H\text{-}^{2d}$ Background) mice (n=4) were prime-boost immunized with FPV-HIV/VV-HIV (control vaccine). At 2 weeks post-booster immunization, $CD8^+$ T cells from splenocytes were negatively enriched and stimulated with $K^dGag_{197\text{-}205}$ peptide for 16 hrs. RNA was isolated, cDNA was synthesized and real-time PCR was performed to evaluate the fold change in the mRNA levels compared to unstimulated background control. Data show the mRNA fold change of IFN-γ and IL-17A compared to the unstimulated control normalized against L32 expression as described in materials and methods. These experiments were repeated twice and similar trends were observed.*

**IL-17A**


300
200
100
0
Fold change
BALB/c
IL-13-/-
IL-4-/-
STAT6-/-

**IFN-γ**

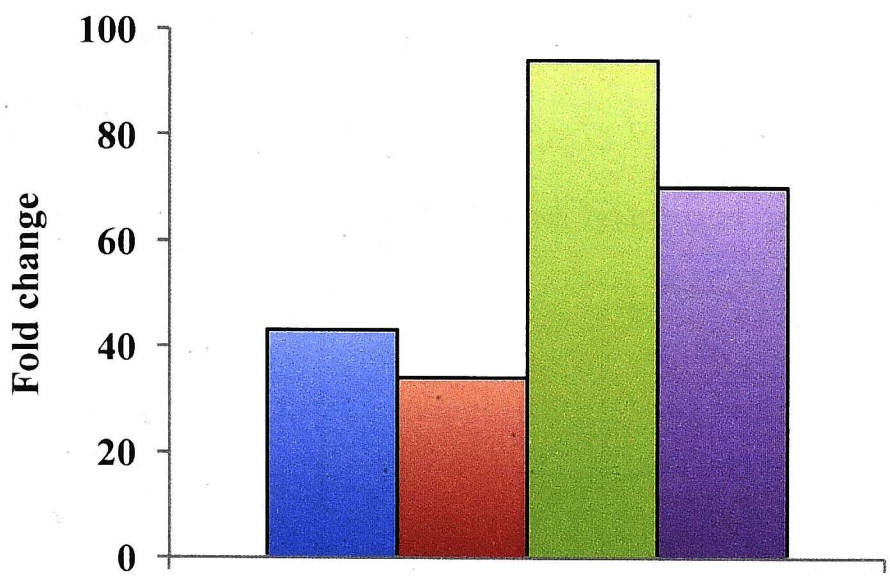
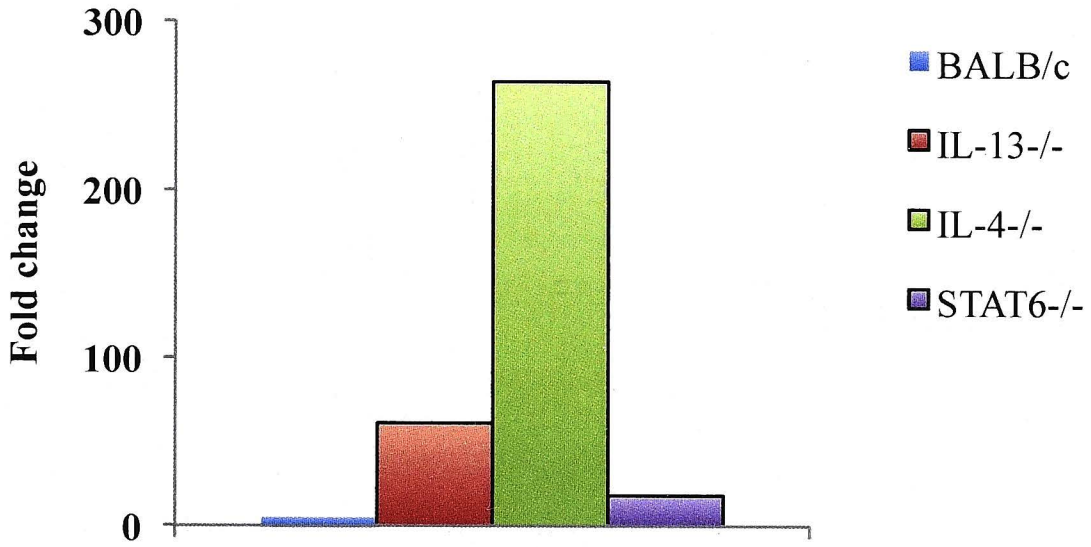

100
80
60
40
20
0
Fold change

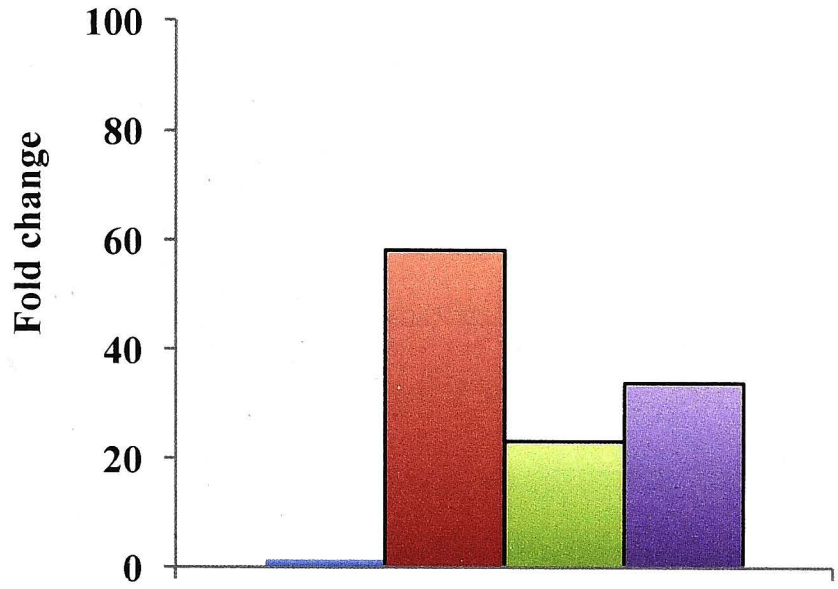
**Fig 4.8: Evaluation of IL-17A and granzyme-B mRNA expression in BALB/c, IL-4-/-, IL-13-/-, and STAT6-/- mice following prime-boost immunization**

*BALB/c, IL-13-/-, IL-4-/- and STAT6-/- (H-$^{2d}$ Background) mice (n=4) were prime-boost immunized with FPV-HIV/VV-HIV (control vaccine). At 2 weeks post-booster immunization, $CD8^{+}$T cells were negatively enriched and stimulated with $K^{d}Gag_{197\text{-}205}$ peptide for 16 hrs. RNA was isolated, cDNA was synthesized and real-time PCR was performed to evaluate the fold change in the mRNA levels compared to unstimulated background control. Data show the mRNA fold change of IL-17A and granzyme-B compared to the unstimulated control normalized against L32 expression. These experiments were repeated twice and similar trends were observed.*

**IL-17A**




**Granzyme-B**



## 4.7 Discussion

Previous studies have shown that IL-6 and TGF-β directs the induction of IL-17A expression in $CD4^{+}$Th17 cells [182, 183]. Current data clearly demonstrated that there is an elevated IL-6 and TGF-β mRNA expression in IL-4-/-, IL-13-/- and STAT6-/- mice compared to BALB/c. However, IL-4-/- mice showed the highest TGF-β and IL-6 mRNA expressions compared to other groups tested. Hence, data indicate that the induction of IL-17A expression in HIV-specific $CD8^{+}$ T cells is strongly regulated by TGF-β and IL-6 in an IL-4 dependent manner (Table 4.1).

Studies by Huber *et al.* have shown that, TGF-β can enhance Th17 programming by inducing ROR-γt, ROR-α, IL-23R expression in $CD8^{+}$ T cells in the presence of IL-6 and IL-21 [181, 184]. Current data showed that ROR-γt mRNA copy numbers were higher in IL-4-/-, IL-13-/- and STAT6-/- mice compared to BALB/c control. Although, ROR-γt is the master transcriptional factor involved in the regulation of IL-17A [177], current data clearly indicate that ROR-γt plays a minor role in regulating IL-17A expression in HIV-specific $CD8^{+}$ T cells compared to TGF-β.

Interestingly, the mRNA copy numbers of TGF-β, IL-6, ROR-γt, IL-17A mRNA were increased at 16 hrs of $K^{d}Gag_{197\text{-}205}$ peptide stimulation compared to 4 hrs in all the KO groups tested. This could possibly be due to the increased rate of transcription at 16 hrs. Previously in chapter 3 at the protein level, the expression of ROR-γt was increased at 6 hrs of $K^{d}Gag_{197\text{-}205}$ peptide stimulation. The mRNA data showed that there was less ROR-γt mRNA expressions at 4 hrs compared to 16 hrs. This suggests that most likely the ROR-γt transcription occurs between 4 hrs and 6 hrs of peptide stimulation. Thus, evaluating the mRNA expression kinetics at several time points (i.e. 5, 6, 7, 8 hrs) may provide better understanding of the IL-17A kinetic profile in HIV-specific $CD8^{+}$ T cells.

Although IL-23 has been demonstrated in the proliferation and persistence of IL-17A production in $CD4^{+}$Th17 cells, following HIV-1 prime-boost immunization, current data showed no observable increase in the expression of IL-23a in HIV-specific $CD8^{+}$ T cells in any of the groups tested. Data suggest that unlike $CD4^{+}$ T cells [20, 21, 67], IL-23 is not required for the induction of IL-17A in HIV-specific $CD8^{+}$ T cells.

The relative expression of IL-17A mRNA copy numbers were higher in IL-4-/-, IL-13-/- and STAT6-/- mice compared to BALB/c control mice. These results were highly

consistent with the findings by He *et al.* where IL-4-/-, IL-13-/- and IL-4/IL-13-/- mice showed elevated IL-17A mRNA expression level by Th17 cells upon OVA sensitization [69]. The current IL-17A mRNA expression profile in KO mice was similar to the protein expression data showing IL-4-/- > IL-13-/- > STAT6-/- > BALB/c mice (table 4.1). This further confirmed that IL-17A expression by HIV-specific $CD8^+$ T cells could mainly be IL-4 dependent and not IL-13.

Previous studies have shown that, both T-bet and eomesodermin (Eomes) (Th1 transcription factor) can regulate the expression of ROR-γt and IL-17A expression [185]. Current data clearly indicated that both T-bet and GATA3 did not significantly influence the transcriptional regulation of IL-17A expression in HIV-specific $CD8^+$ T cells, suggesting that IFN-γ (regulated by T-bet) may not play a role in dampening IL-17A expression. For example both ROR-γt and T-bet were highest in IL-4-/- mice. Chapter-3 results have also shown that IL-4-/- mice produced highest $CD8^+IL\text{-}17^+IFN\text{-}\gamma^+$ cells. Interestingly, the level of GATA3 mRNA expressions was higher in all the KO groups tested. It is well established that GATA3 play a important role in IL-4/IL-13 regulation [186] [187]. Studies by Ouyang *et al.* have shown that GATA3 activation of T cells can occur independent of STAT6 signaling [188] and also, it has been well studied that the IL-4 and IL-13 can compensate each other in the absence of one to initiate the Th2 mediated immune response [189-191].

Previously, Tajima *et al.* have shown that naïve $CD8^+$ T cells from OT-1 TCR transgenic mice produced higher IFN-γ and granzyme-B mRNA expression under Th1 polarizing conditions and IL-17A and ROR-γt under Th17 polarizing conditions suggesting that Th1 cells were strongly cytotoxic whereas Th17 cells most likely not cytotoxic [171, 181]. Current data indicated that IFN-γ and IL-17A were highest in IL-4-/- mice (chapter 3). However, granzyme-B and IL-17A expressions showed an inverse relationship with IL-13 and IL-4 KO mice suggesting that HIV-specific $CD8^+$ T cells obtained from IL-13 KO mice could possibly be highly cytotoxic compared to $CD8^+$ T obtained from IL-4 KO mice. In our laboratory, Quah *et al.* have designed a novel assay to look at the cytotoxicity and most likely; this assay could evaluate this function of IL-13 and IL-4 KO mice, which warrants further investigation.

In summary, current data indicate that the IL-17A expression at the mRNA level was regulated mainly by TGF-β and IL-6, and to a lesser extent ROR-γt but not IL-23a.

Following HIV-specific peptide stimulation, T-bet and GATA3 did not play any role in regulating IL-17A expression in HIV-specific $CD8^+$ T cells. Data indicated that in wild type BALB/c mice, IL-4, IL-13 and STAT6 down regulate the IL-17A mRNA expression by HIV-specific $CD8^+$ T cells. In contrast, IFN-γ did not dampen the IL-17A mRNA expression by HIV-specific $CD8^+$ T cells. Granzyme-B and IL-17A expressions were inversely correlated in IL-13 and IL-4 KO mice suggesting that in IL-13-/- mice, HIV-specific $CD8^+$ T cells could be more cytotoxic than IL-4 -/- mice which needs to be confirmed using the cytotoxicity assay.

# CHAPTER 5: Evaluate the IL-17A expression by HIV-specific $CD8^+$ T cells in response to the novel IL-13 inhibitor vaccine

## 5.1 Introduction:

Studies in our laboratory have shown that i.n/i.m prime-boost immunization can induce high magnitude (measured by IFN-γ) and high avidity HIV specific effector and memory $CD8^+$ T cell responses in BALB/c mice [144]. Studies using IL-13-/-, IL-4-/- mice, have further substantiated that IL-13 and IL-4 are involved in regulating the avidity of HIV-specific CTLs [165]. Recently, Ranasinghe *et al.* have developed an HIV pox-viral based vaccine that co-expresses HIV gag/pol and IL-13 soluble receptor (IL-13Rα2), which can temporarily inhibit the expression of IL-13 *in vivo*. This IL-13 inhibitor vaccine strategy (FPV-HIV IL-13Rα2/VV-HIV IL-13Rα2) has shown to enhance both the magnitude and avidity of HIV-specific $CD8^+$ T cells compared to the control vaccine (FPV-HIV/VV-HIV) (Ranasinghe *et al.* submitted May 2012). Various studies have shown that IL-17A is involved in protective immunity [27, 192]. As chapters 3 and 4 have clearly shown that IL-4 and IL-13 can modulate IL-17A expression, in this study, the expression of IL-17A was evaluated at acute, effector, memory stages and following influenza-HIV challenge using the novel IL-13 inhibitor vaccine to further establish whether IL-17A expression was a hallmark of protective immunity and some how involved in modulating T cell avidity.

## 5.2 Evaluation of IFN-γ and IL-17A expression by HIV-specific effector $CD8^+$ T cells following novel IL-13 inhibitor vaccine:

To evaluate the expression of IFN-γ and IL-17A following novel IL-13 inhibitor vaccine, firstly, mice (n=5-8) were prime-boost immunized with either FPV-HIV/VV-HIV (control vaccine) or FPV-HIV IL-13Rα2/VV-HIV IL-13Rα2 (IL-13 inhibitor vaccine) as described in methods. At 14 days post booster immunization, the expression of IFN-γ and IL-17A by $CD8^+$ T cells were evaluated by intracellular cytokine staining and IL-17A ELIspot.

Results indicated that the IL-13 inhibitor vaccine generated significantly higher expression of IFN-γ compared to the control vaccine (Fig 5.1), which was consistent with previous studies in our laboratory (Ranasinghe *et al.* submitted in May 2012). When IL-17A expression was evaluated, data clearly indicated that, at 14 days post booster immunization, IL-13 inhibitor vaccine could induce elevated $K^dGag_{197\text{-}205}$ specific $CD8^+$ T cells that expressed IL-17A. Interestingly, control vaccine showed no detectable IL-17A expression by HIV-specific $CD8^+$ T cells (Fig 5.2). In this study, the

responses observed by IL-13 inhibitor vaccine were much lower compared to the IL-13-/- mice (chapter 3) possibly due to the transient inhibition by the novel IL-13 inhibitor vaccine unlike the KO mice.

To further confirm the IL-17A expression, ELIspot was also performed by enriching the $CD8^+$ T cells as described previously in chapter 2. The IL-13 inhibitor vaccine results were consistent with the IL-17A intracellular cytokine staining data, where elevated number of IL-17A SFU were observed in the spleen following $K^dGag_{197-205}$ peptide stimulation. However, unlike ICS, where total splenocytes were used control vaccine also showed around twenty IL-17A SFU produced by $CD8^+$ T cells (Fig 5.3).

**5.3 Evaluation of lung specific IFN-γ and IL-17A expression by HIV-specific $CD8^+$ T cells following novel IL-13 inhibitor vaccine:**

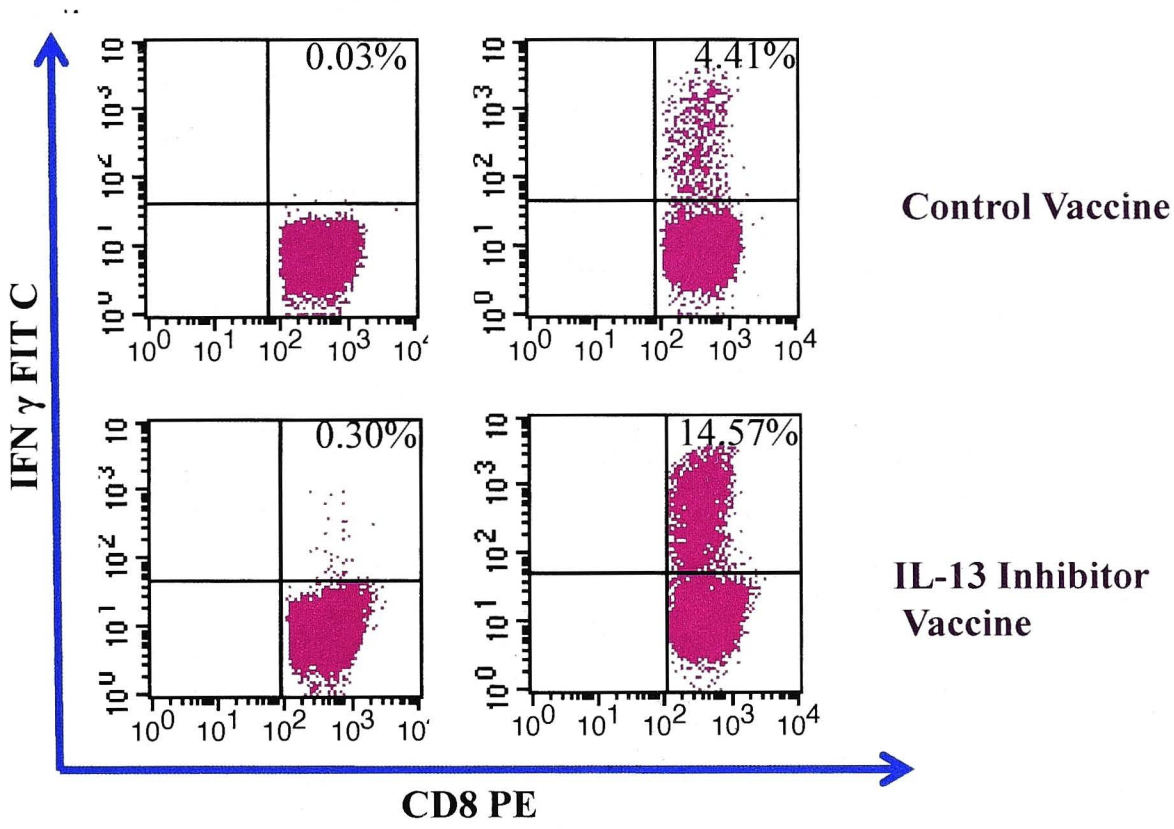
The above data (Fig 5.1 and 5.2) indicated that the expression of splenic $CD8^+$ IFN-γ and IL-17A production was higher in the IL-13 inhibitor vaccine compared to the control vaccine. Therefore, the expressions of IFN-γ and IL-17A by $CD8^+$ T cells in the lung (n=5-8) were also examined to assess whether the vaccines induced immunity in the mucosae. Results indicated that the IFN-γ and IL-17A production was enhanced in lung at 14 days post booster immunization similar to that of spleen compared to the control vaccine (Fig 5.4 and 5.5).

Then the total number of IL-17A SFU in the lung was further examined by ELIspot, (note that the cells were not enriched due to small sample size) and results clearly showed that the total number IL-17A expression by lung specific T cells following $K^dGag_{197-205}$ peptide stimulation were higher in IL-13 inhibitor vaccine compared to the control vaccine (Fig 5.6). However, data indicated that unlike the spleen where $CD8^+$ T cells were negatively isolated, there was spontaneous IL-17A production by cells other than $CD8^+$ T cells as elevated IL-17 SFU were observed in the unstimulated control. These background numbers were higher in the novel IL-13 inhibitor vaccine compared to the control vaccine.

**Fig 5.1: Evaluation of IFN-γ expression by systemic HIV-specific effector $CD8^+$ T cells following novel IL-13 inhibitor vaccine**

*BALB/c ($H\text{-}^{2d}$ Background) mice were prime-boost immunized with FPV-HIV/VV-HIV (control vaccine) or FPV-HIV IL-13Rα2/VV-HIV IL-13Rα2 (IL-13 inhibitor vaccine). At 14 days post-booster immunization, splenocytes were prepared as described in materials and methods. 4 x $10^6$ cells were stimulated with $K^dGag_{197\text{-}205}$ peptide for 16-20 hrs in the presence of brefeldin-A to evaluate IFN-γ expression by intracellular cytokine staining. a) The FACS plots indicate representative animals from each group. In all FACS plots, the upper right quadrant (R3) indicates gates R1+R2 (see fig 3.1) and the numbers indicate the percentage of $CD8^+$ T cells producing IFN-γ. b) The graph indicates the percentage of $CD8^+$T cells expressing IFN-γ (n=5-8 mice) where the stimulated samples were subtracted from the unstimulated background. Data are representative of three independent experiments and error bars represents standard error of the mean (SEM). p values were calculated using two tailed un-paired student's t test *p=0.0001.*

*(a)*


Unstimulated
Stimulated
0.03%
4.41%
0.30%
14.57%
Control Vaccine
IL-13 Inhibitor Vaccine
IFN γ FIT C
CD8 PE

*(b)*

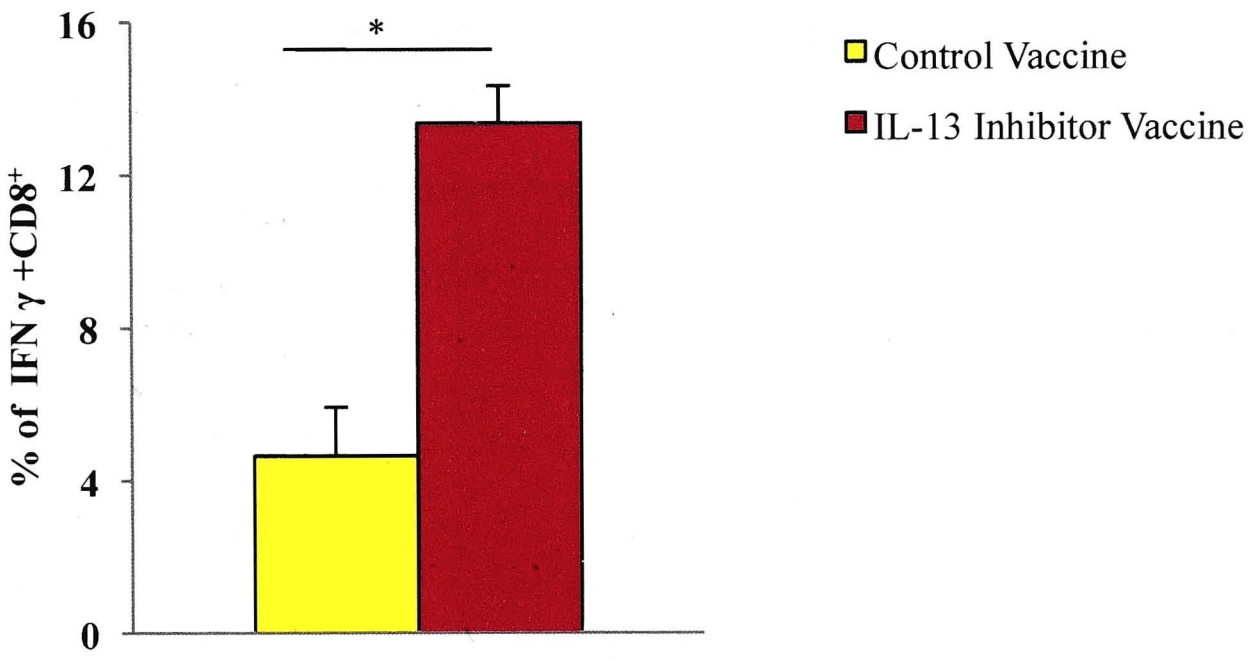
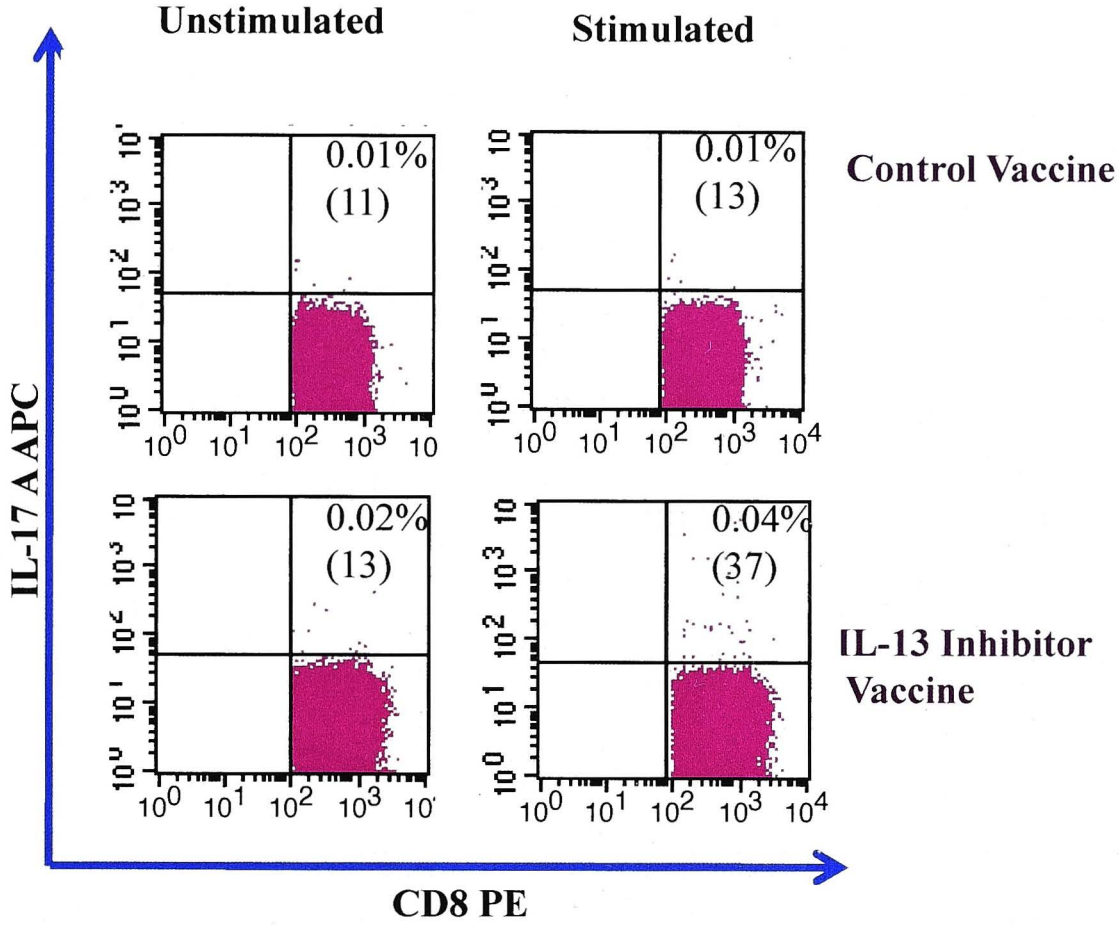

16
12
8
4
0
% of IFN γ +CD8+
*
Control Vaccine
IL-13 Inhibitor Vaccine

**Fig 5.2: Evaluation of IL-17A expression by systemic HIV-specific effector $CD8^+$ T cells following novel IL-13 inhibitor vaccine**

*BALB/c mice were prime –boost immunized with FPV-HIV/VV-HIV (control vaccine) or FPV-HIV IL-13Rα2/VV-HIV IL-13Rα2 (IL-13 inhibitor vaccine). At 14 days post booster immunization, splenocytes were prepared as described in materials and methods. 4 x $10^6$ cells were stimulated with $K^dGag_{197-205}$ peptide for 16-20 hrs in the presence of brefeldin-A to evaluate IL-17A expression by intracellular cytokine staining. a) The FACS plots indicate representative animals from each group. In all FACS plots the upper right quadrant (R3) indicates gates R1+R2 and the numbers indicate IL-17A producing $CD8^+$ T cells as a percentage (top) and the number of events (bottom within brackets). b) The graph indicates the percentage of $CD8^+$ T cells expressing IL-17A (n=5-8 mice) in each group. In all graphs, unstimulated cells were used as a background controls and were subtracted from each sample before plotting the data. The data are representative of three independent experiments and error bars represents standard error of the mean (SEM). p values were calculated using two-tailed un-paired student's t test *p=0.025.*

*(a)*


Unstimulated
Stimulated
0.01%
(11)
0.01%
(13)
Control Vaccine
0.02%
(13)
0.04%
(37)
IL-13 Inhibitor Vaccine
IL-17A APC
CD8 PE

*(b)*

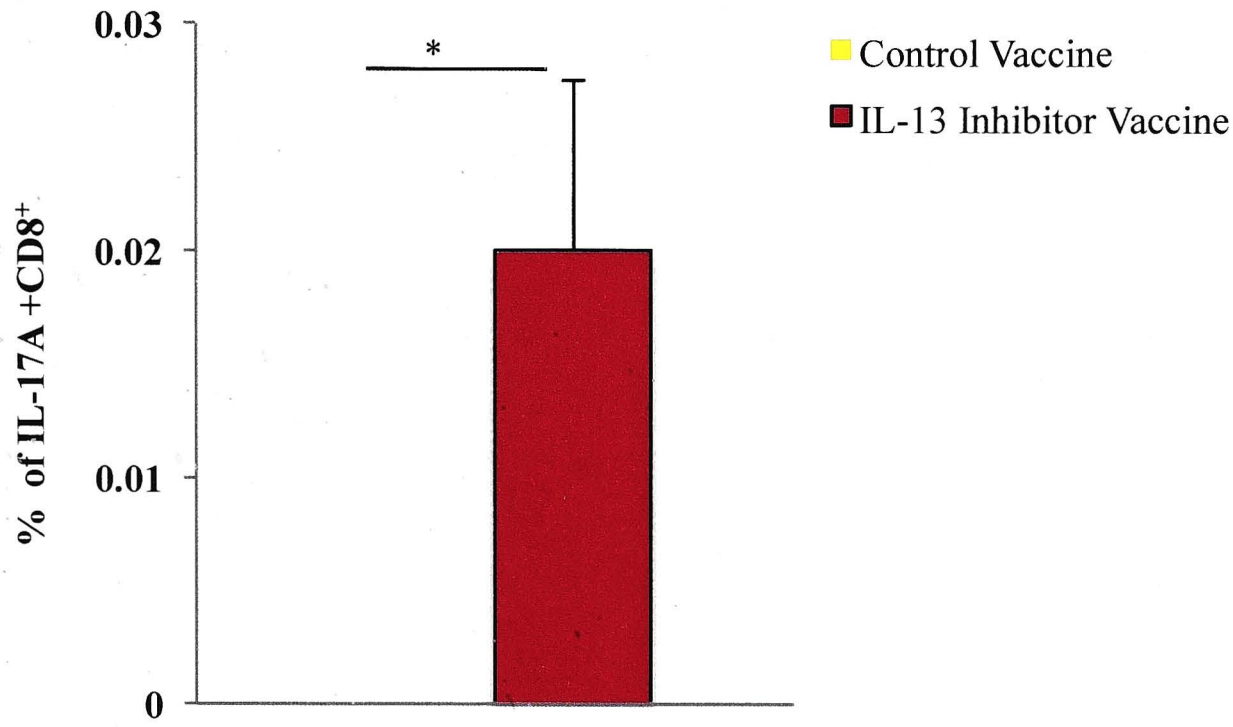
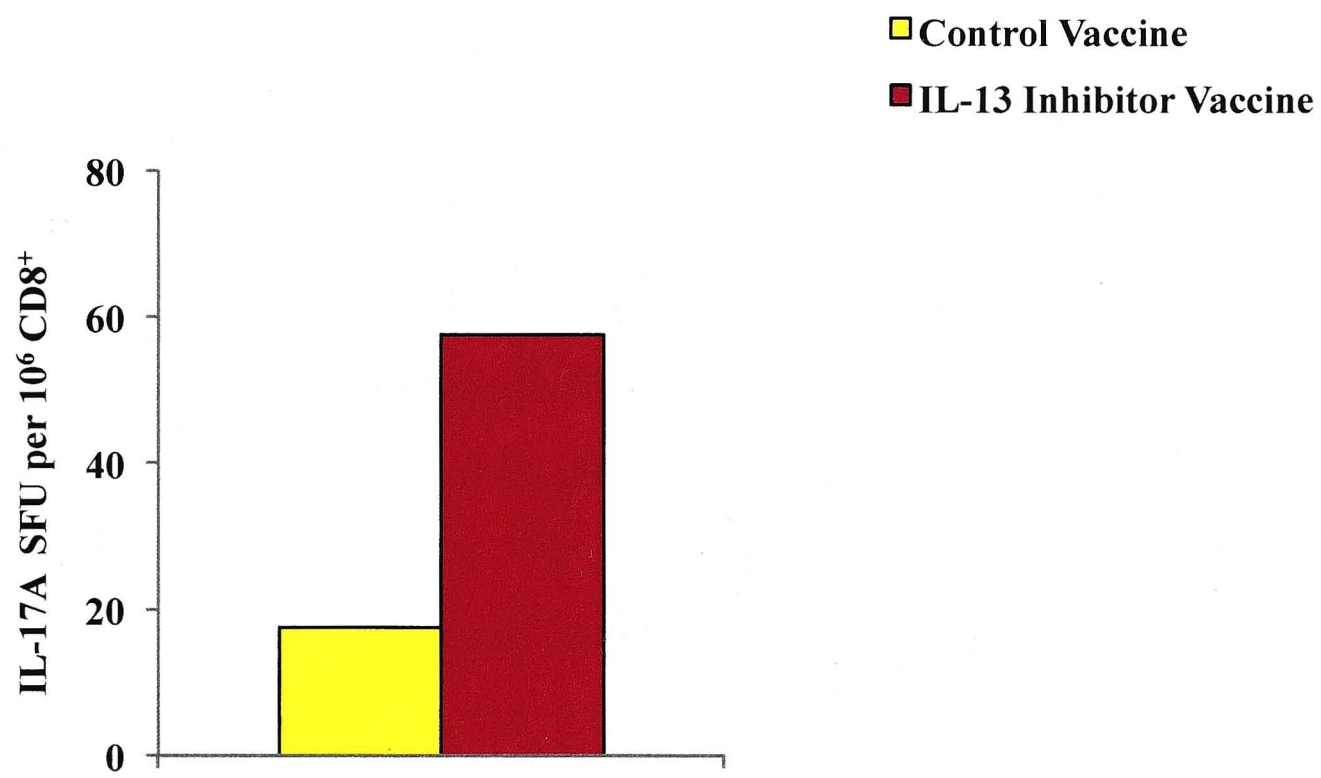

0.03
0.02
0.01
0
% of IL-17A +CD8+
*
Control Vaccine
IL-13 Inhibitor Vaccine

**Fig 5.3: Evaluation of splenic IL-17A expression following novel IL-13 inhibitor vaccine using ELIspot compared to ICS**

*BALB/c (H-$^{2d}$ Background) mice were prime-boost immunized with FPV-HIV/VV-HIV (control vaccine) or FPV-HIV IL-13Rα2/VV-HIV IL-13Rα2 (IL-13 inhibitor vaccine). Splenocytes were harvested and IL-17A expression was measured by a) IL-17A ELIspot using negatively isolated $CD8^+$T cells as described previously and b) intracellular cytokine staining. The graphs represent a) the total number IL-17 SFU per $10^6$ $CD8^+$ T cells measured by ELIspot, compared to the number of IL-17 cells per $10^6$ $CD8^+$ T cells measured by ICS. Unstimulated cells from each sample were used as background controls. The data represent mean plus standard error of the mean (SEM). p values were calculated using two-tailed un-paired student's t test *p=0.037.Data are representative of three independent experiments.*

*(a) ELIspot*




*(b) ICS*

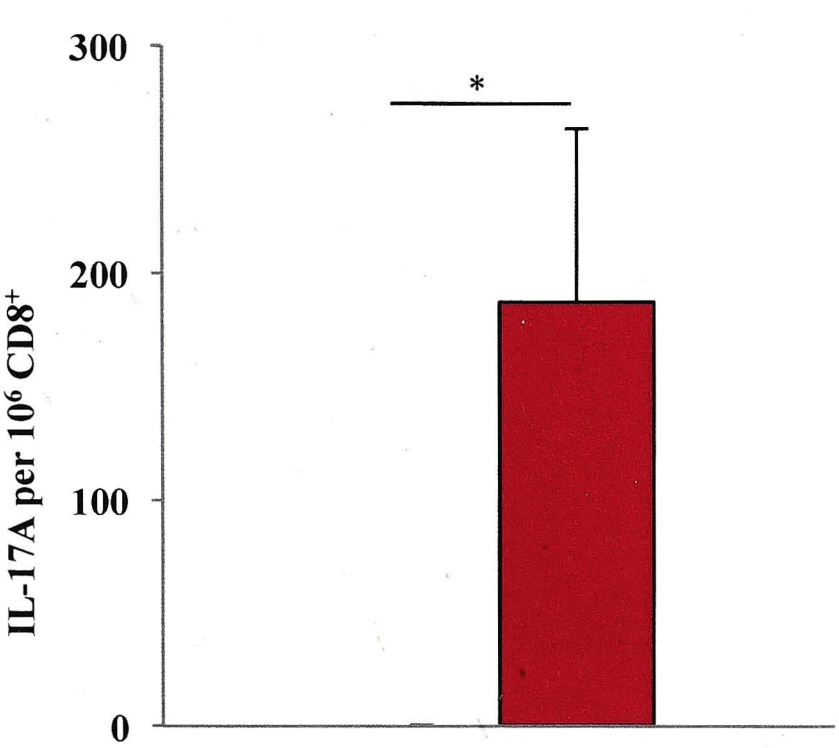
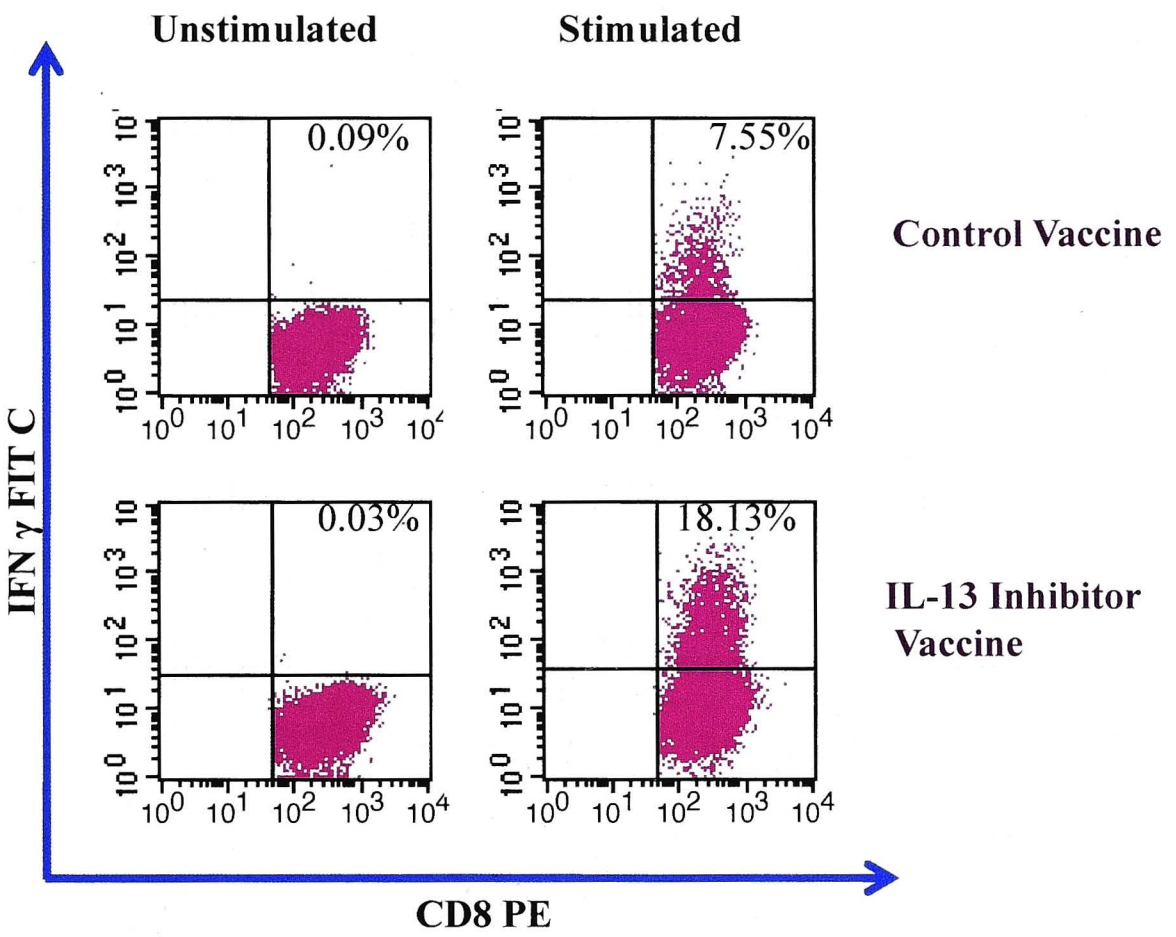


**Fig 5.4: Evaluation of IFN-γ expression by lung HIV-specific effector $CD8^+$ T cells following novel IL-13 inhibitor vaccine**

*BALB/c (H-$^{2d}$ Background) mice (n=5) were prime-boost immunized with FPV-HIV/VV-HIV (control vaccine) or FPV-HIV IL-13Rα2/VV-HIV IL-13Rα2 (IL-13 inhibitor vaccine). At 14 days post booster immunization, lung cells were prepared as described in materials and methods and 4 x $10^6$ cells were stimulated with $K^dGag_{197-205}$ peptide for 16-20 hrs in the presence of brefeldin-A and IFN-γ expression was evaluated by intracellular cytokine staining. a) The FACS plots indicate data pooled from animals (n=5 mice). In all FACS plots, the upper right quadrant (R3) indicates gates R1+R2 and the numbers in the quadrant indicate the percentage of $CD8^+$ T cells producing IFN-γ. b) The graph shows the representative data from two experiments where the samples were pooled from each group.*

*(a)*


Unstimulated
Stimulated
0.09%
7.55%
0.03%
18.13%
Control Vaccine
IL-13 Inhibitor Vaccine
IFN γ FIT C
CD8 PE

*(b)*

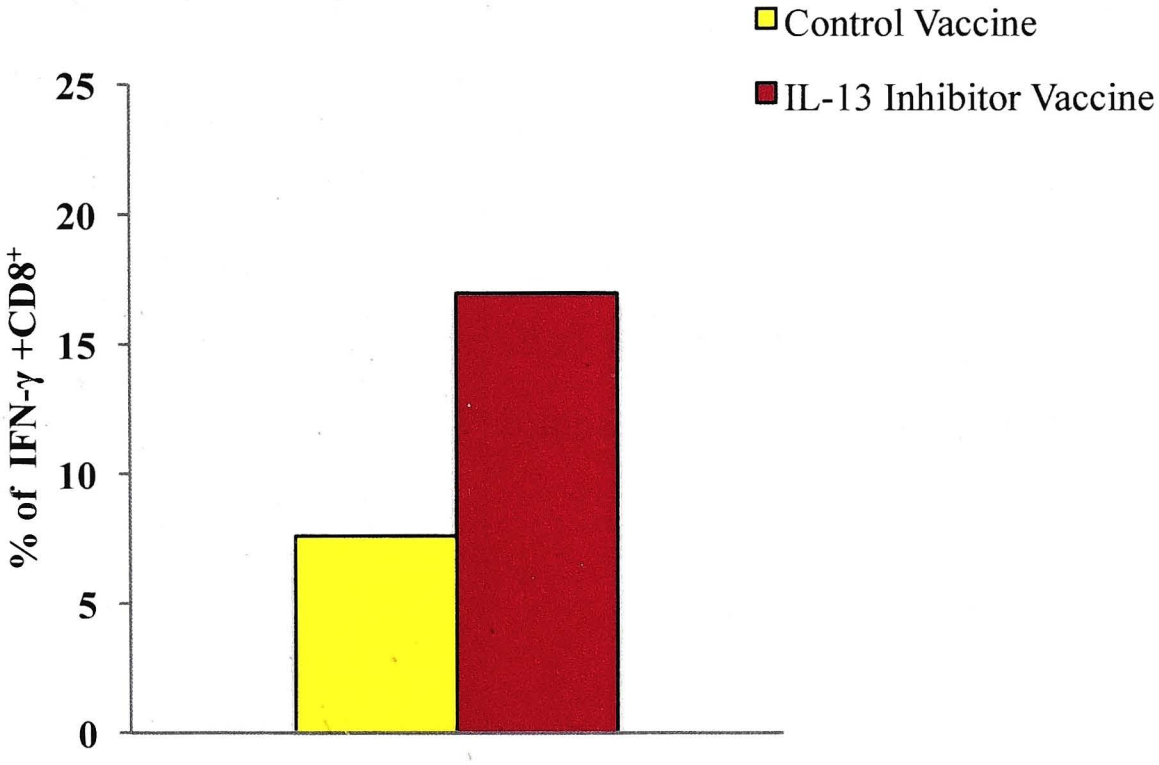
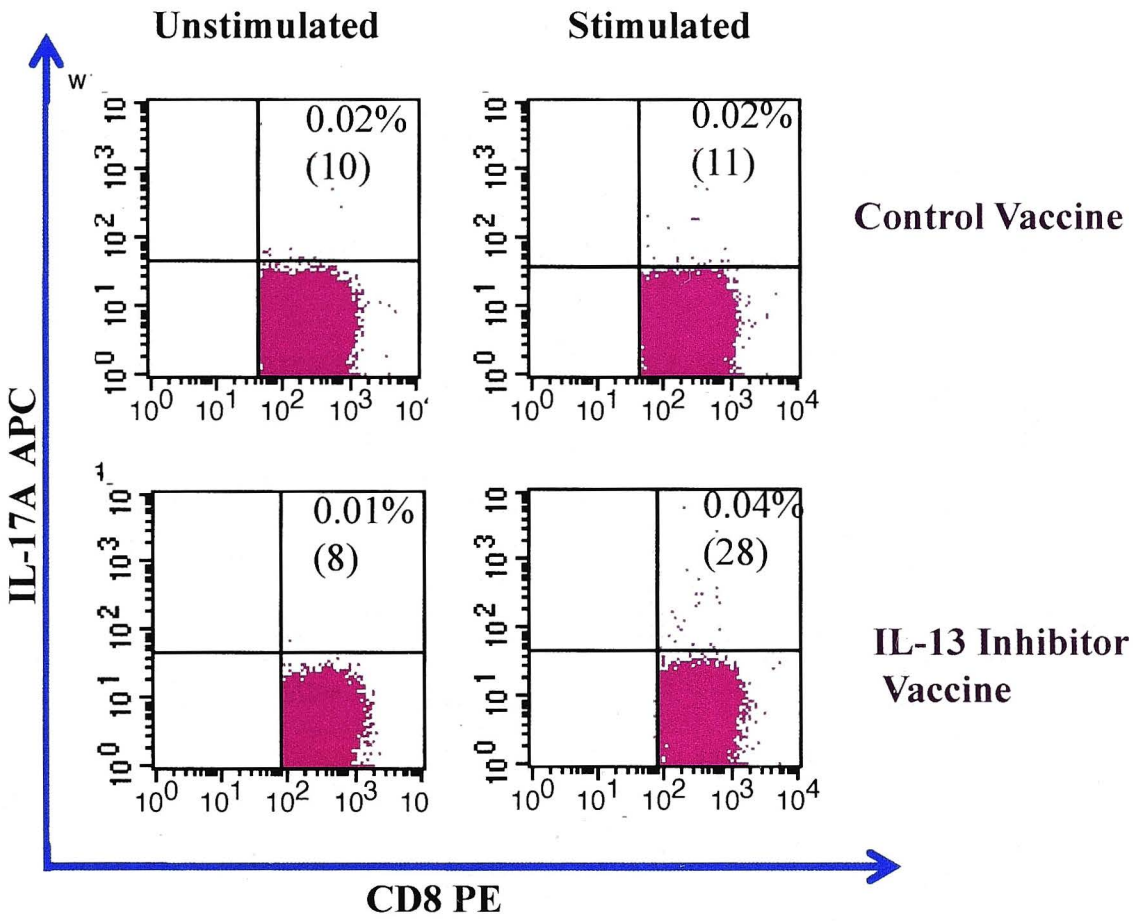

Control Vaccine
IL-13 Inhibitor Vaccine
% of IFN-γ +CD8+
0
5
10
15
20
25

**Fig 5.5: Evaluation of IL-17A expression by lung HIV-specific effector $CD8^+$ T cells following novel IL-13 inhibitor vaccine**

*BALB/c (H-$^{2d}$ Background) mice (n=5) were prime-boost immunized with FPV-HIV/VV-HIV (control vaccine) or FPV-HIV IL-13Rα2/VV-HIV IL-13Rα2 (IL-13 inhibitor vaccine). At 14 days post booster immunization, lung cells were prepared as described in materials and methods. 4 x $10^6$ cells were stimulated with $K^dGag_{197-205}$ peptide for 16-20 hrs in the presence of brefeldin-A and IL-17A expression was evaluated by intracellular cytokine staining. a) The FACS plots indicate pooled animals from each group. In all FACS plots, the upper right quadrant (R3) indicates gates R1+R2 and the numbers indicate IL-17A producing $CD8^+$ T cells as a percentage (top) and the number of events (bottom within brackets). Unstimulated cells from each sample were used as background control. b) The graph indicates representative data from two independent experiments. In all graphs, unstimulated cells were used as a background controls and were subtracted from each sample before plotting the data.*

*(a)*


Unstimulated
Stimulated
0.02%
(10)
0.02%
(11)
Control Vaccine
0.01%
(8)
0.04%
(28)
IL-13 Inhibitor Vaccine
IL-17A APC
CD8 PE

*(b)*

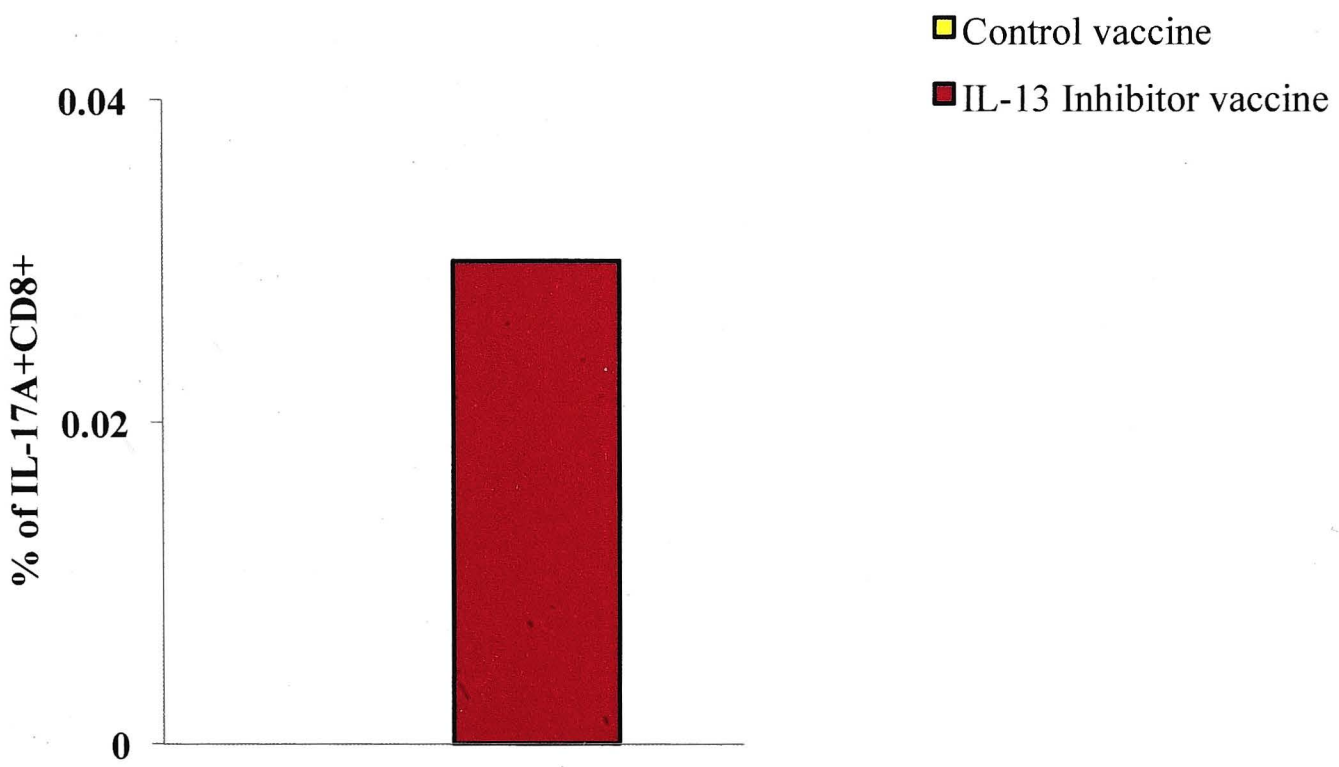
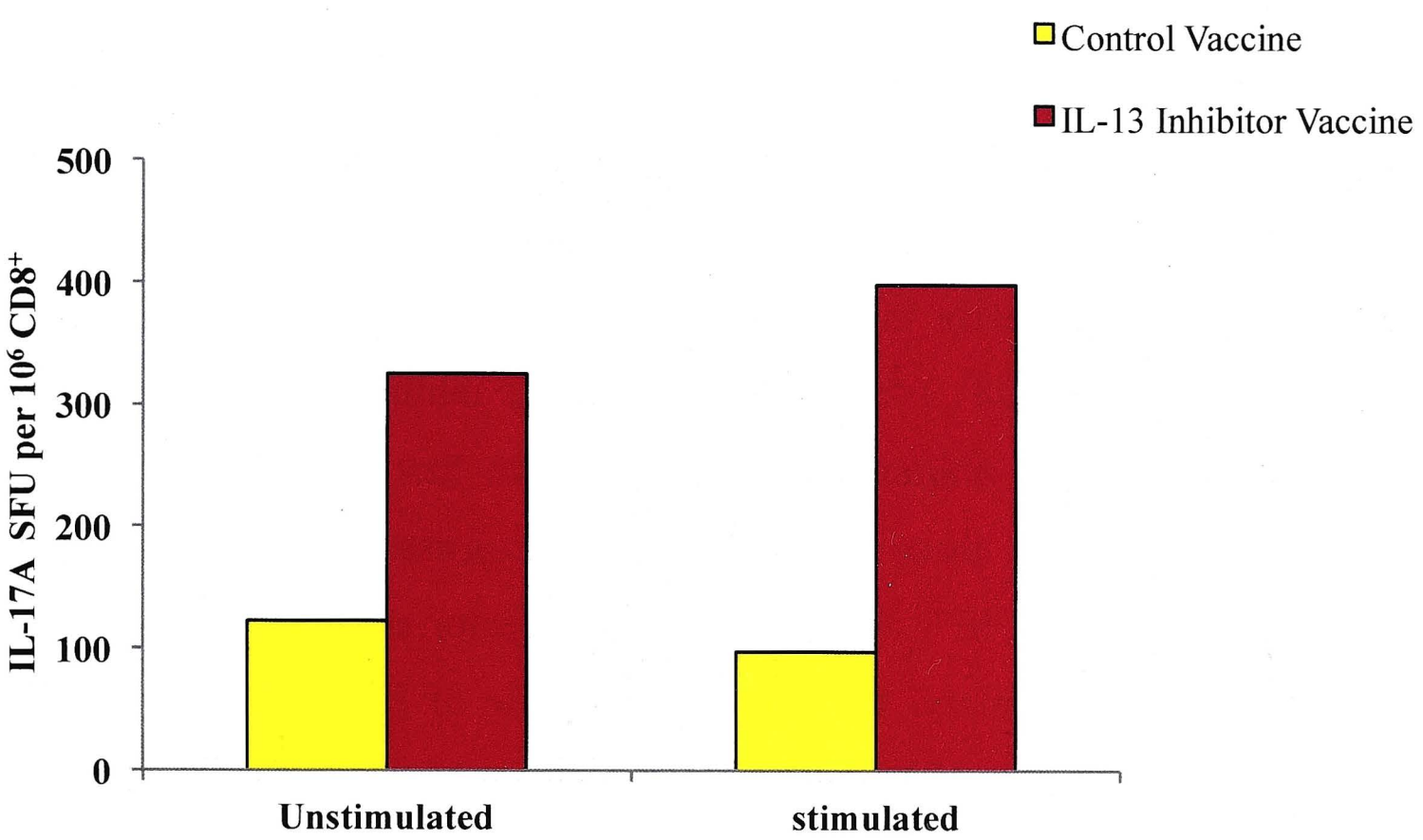

Control vaccine
IL-13 Inhibitor vaccine
0.04
0.02
0
% of IL-17A+CD8+

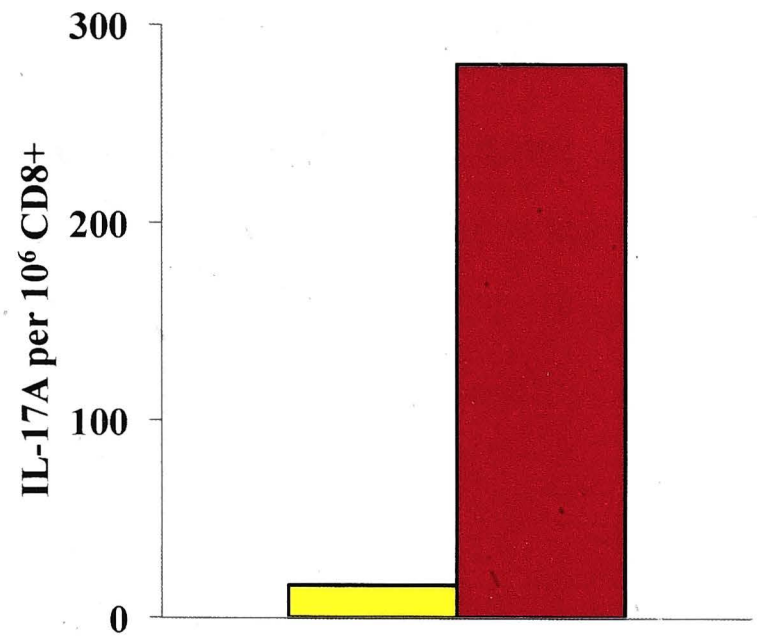
**Fig 5.6: Evaluation of lung specific IL-17A expression following IL-13 Inhibitor vaccine using ELIspot compared to ICS**

*BALB/c (H-$^{2d}$ Background) mice (n=5) per group were prime-boost immunized with FPV-HIV/VV-HIV (control vaccine) or FPV-HIV IL-13Rα2/VV-HIV IL-13Rα2 (IL-13 inhibitor vaccine). At 14 days post booster immunization, lung samples were prepared and stimulated with $K^dGag_{197-205}$ peptide as described previously and IL-17A expression was measured by a) IL-17A ELIspot and b) intracellular cytokine staining. The graphs represent a) the total number IL-17 SFU per $10^6$ $CD8^+$ T cells measured by ELIspot compared to the number of IL-17 cells per $10^6$ $CD8^+$ T cells measured by ICS. Unstimulated cells from each sample were used as background controls. Data are representative of two experiments.*

***(a) ELIspot***




***(b) ICS***



### 5.4 Comparison of IL-17A expression by $K^dGag_{197-205}$ specific $CD8^+$ T cells at acute, effector, memory and following influenza-HIV-challenge:

As the IL-17A expression was found to be elevated at 14 days in IL-13 inhibitor vaccine compared to the control vaccine, the expression of IL-17A by $K^dGag_{197-205}$ specific $CD8^+$ T cells at different time intervals following prime-boost immunization was evaluated. Results indicated that at 3 days post booster vaccination, both vaccines induced the lowest IL-17A expression in HIV specific $CD8^+$ T cells compared to 14 days (Fig 5.7). However, at 8 weeks (memory phase), IL-17A expression by $K^dGag_{197-205}$ specific $CD8^+$ T cell was significantly enhanced in IL-13 inhibitor vaccine compared to the control vaccine. To further evaluate the protective role of IL-17A, mice were immunized with both the vaccines and challenged with a model influenza-HIV virus challenge. The data clearly indicated that there was elevated IL-17A expression by $K^dGag_{197-205}$ specific $CD8^+$ T cells following IL-13 inhibitor vaccine compared to the control vaccine or the unimmunized control mice that received the influenza-HIV challenge. Furthermore, these results were also consistent with ELIspot data where higher number of IL-17 SFU were observed at effector stage and following influenza-HIV challenge. In summary, the expression profile of IL-17A following vaccination was found to be influenza-HIV challenge > effector > memory > acute phase (Fig 5.7).

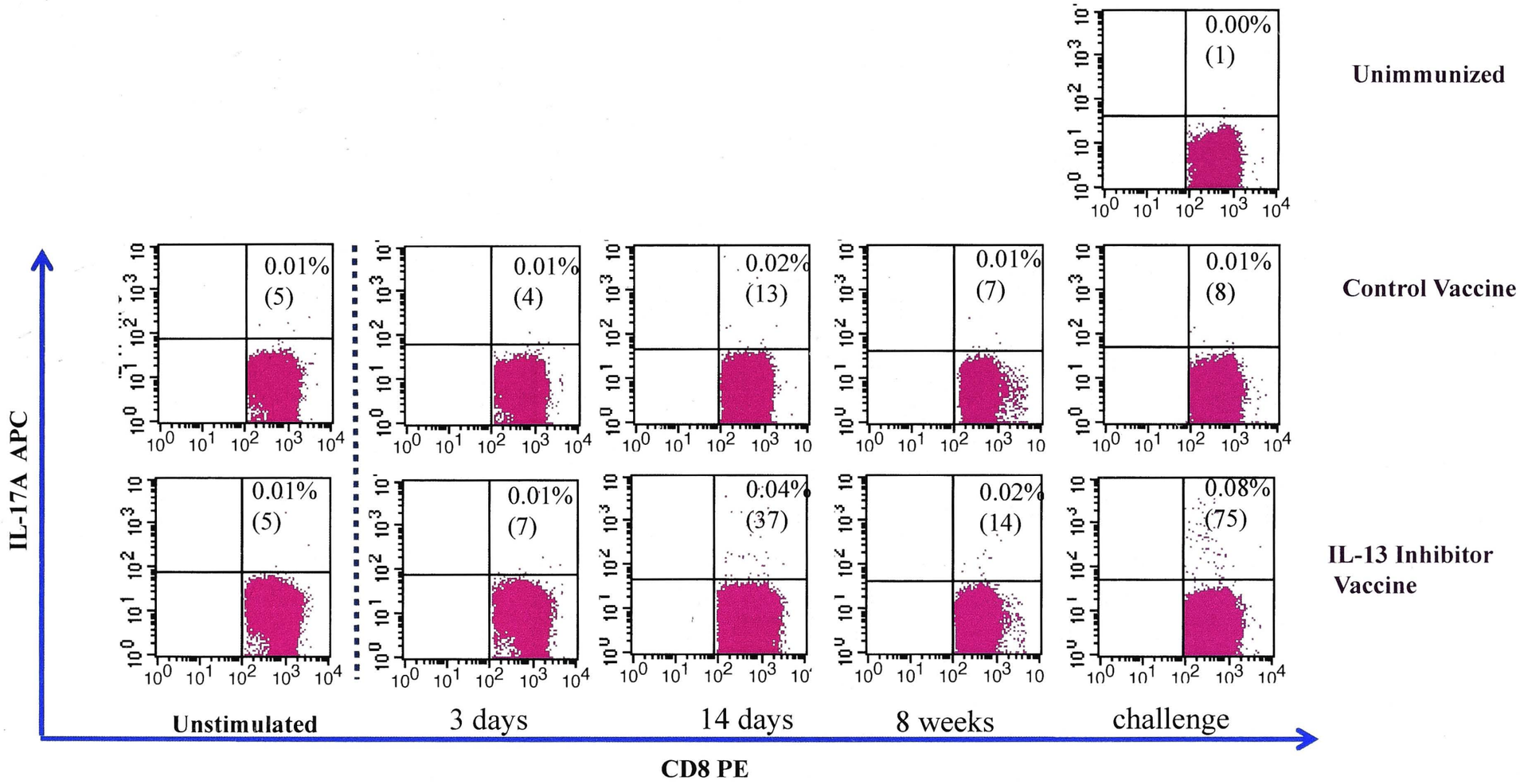
Similarly, when the IL-17A expression was evaluated in the lung, no expression of IL-17A by $K^dGag_{197-205}$ specific $CD8^+$ T cells were observed at 3 days or 8 weeks post booster immunization (Fig 5.8). In contrast, at 14 days and following influenza-HIV challenge, the expression of IL-17A was greatly enhanced (Fig 5.8) in IL-13 inhibitor vaccine compared to the control and unimmunized mice. Unlike the splenic HIV-challenge ELIspot data, lung data did not match what was observed with ICS as higher SFU were detected in the unstimulated lung samples of IL-13 inhibitor vaccine. However, lung ICS data indicated that IL-17A expression by HIV-specific $CD8^+$ T cells were specific to the novel vaccine. Following IL-13 inhibitor vaccine, the IL-17A expression profile in the lung was found to be influenza-HIV challenge > effector > memory $\geq$ acute.

**Fig 5.7: Comparative analysis of IL-17A expression by systemic $K_dGag_{197-205}$ specific $CD8^+$ T cells at acute, effector, memory and following influenza-HIV challenge**

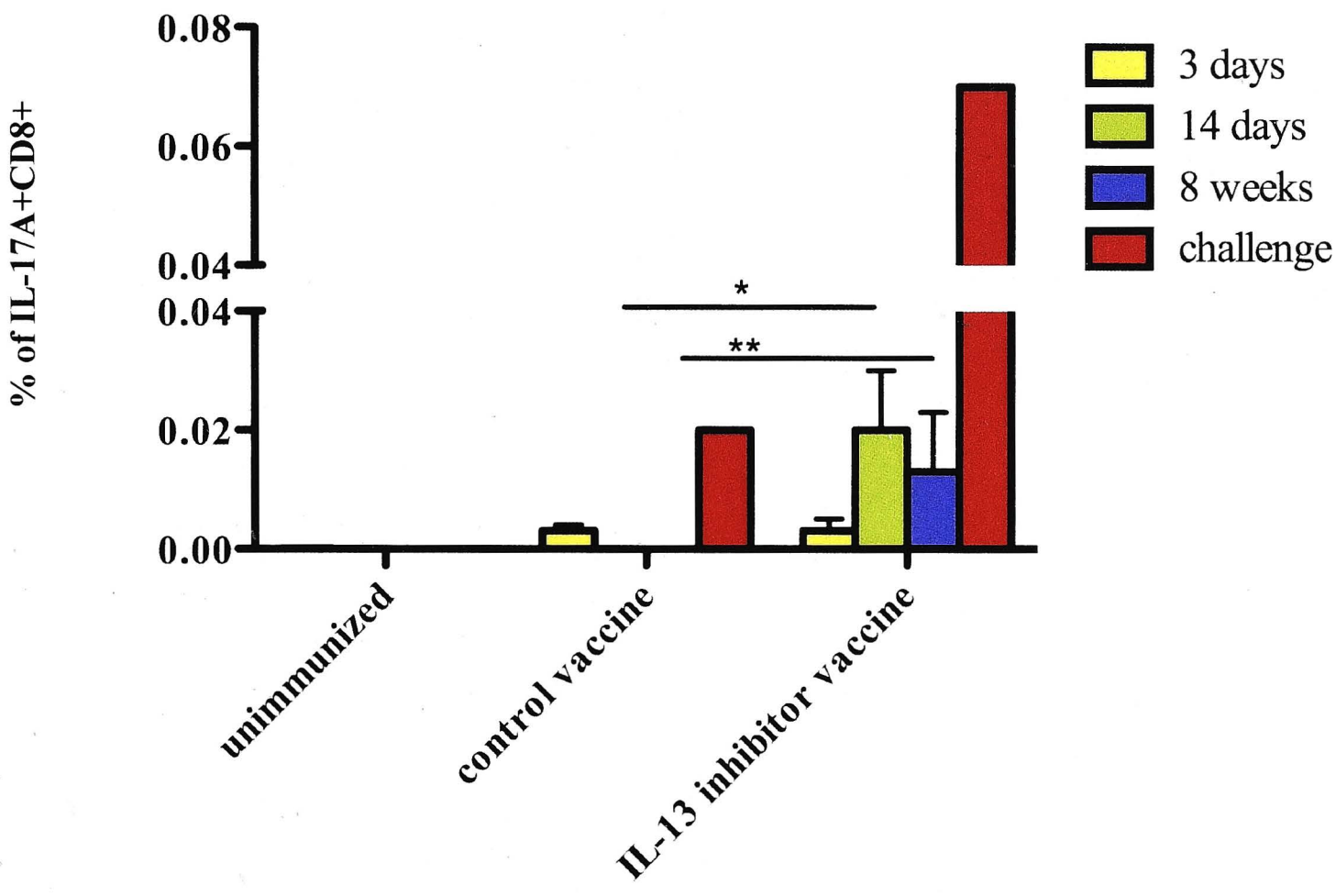
*BALB/c (H-$^{2d}$ Background) mice (n=5-8) were prime-boost immunized with FPV-HIV/VV-HIV (control vaccine) or FPV-HIV IL-13Rα2/VV-HIV IL-13Rα2 (IL-13 inhibitor vaccine). At 3 days, 14 days, 8 weeks and influenza-HIV challenge, spleen samples were harvested and 4 x $10^6$ cells were stimulated with 16-20 hrs of $K^dGag_{197-205}$ peptide stimulation, the expression of IL-17A by $CD8^+$ T cells were evaluated by IL-17A ELIspot and intracellular cytokine staining. The FACS plots indicate representative animals from each group. a) In all FACS plots, the upper right quadrant (R3) indicates R1+R2 and the numbers indicate IL-17A producing $CD8^+$ T cells as a percentage (top) and also the gated number of events (bottom within brackets). b) Graph represents the percentage of $CD8^+$ T cells expressing IL-17A where the unstimulated background value was subtracted. The data represents mean of three independent experiments and error bars indicate standard error of the mean (SEM). p values were calculated using student's two tailed unpaired t test, (*p=0.025) and **p=0.038). c) The graphs indicate the total number IL-17A SFU per $10^6$ $CD8^+$ T cells measured by ELIspot compared to the number of IL-17 cells per $10^6$ $CD8^+$ T cells as measured by ICS. p values were calculated using student's two tailed unpaired t test, (*p=0.037) and **p=0.026).*

*Note: In ICS, Unstimulated values were similar in all experiment time points and the background range was between 0.00-0.02%.*

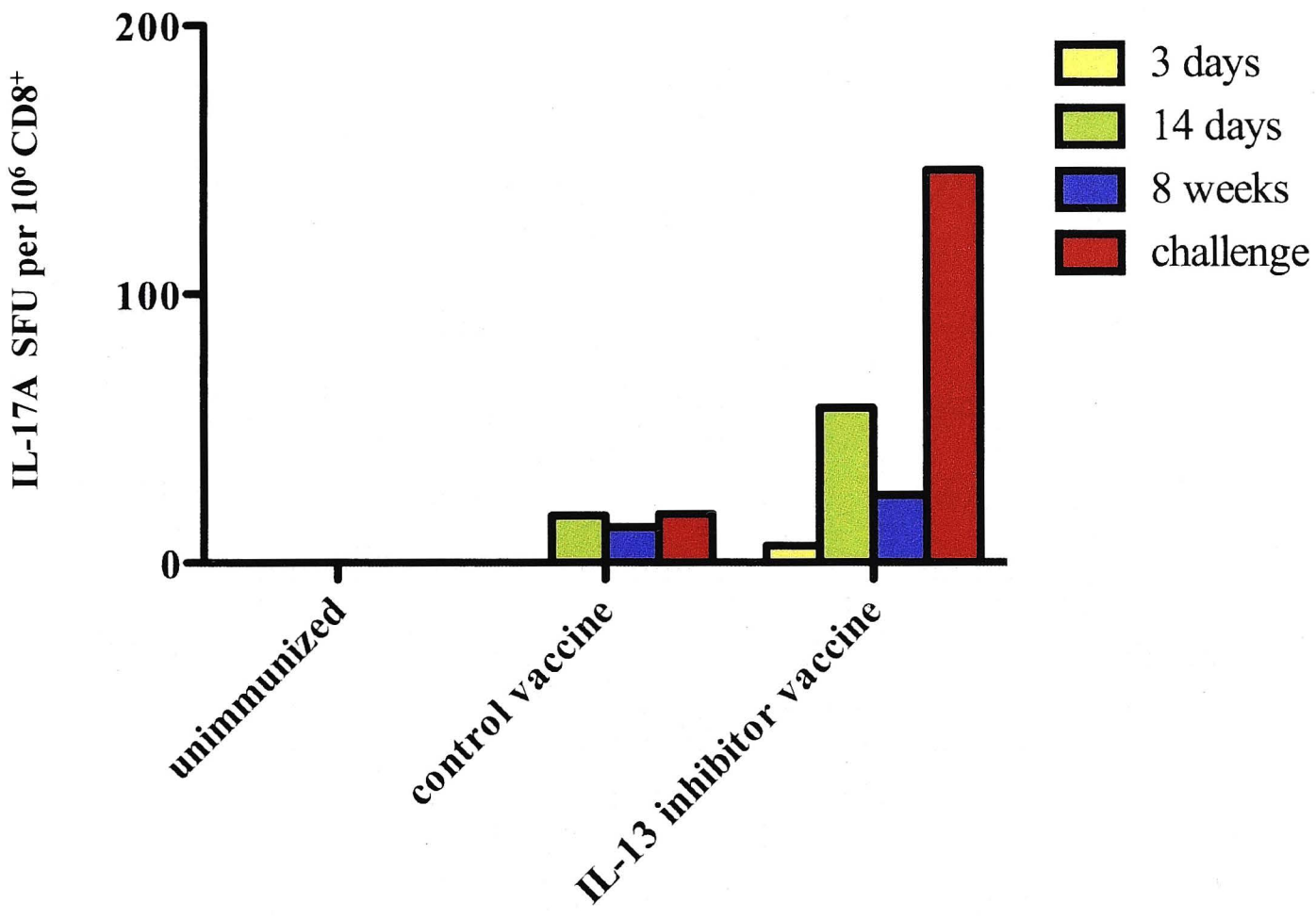
**(a)**


0.00%
(1)
Unimmunized
0.01%
(5)
0.01%
(4)
0.02%
(13)
0.01%
(7)
0.01%
(8)
Control Vaccine
0.01%
(5)
0.01%
(7)
0:04%
(37)
0.02%
(14)
0.08%
(75)
IL-13 Inhibitor Vaccine
IL-17A APC
Unstimulated
3 days
14 days
8 weeks
challenge
CD8 PE

***(b) ICS***


% of IL-17A+CD8+
0.08
0.06
0.04
0.04
0.02
0.00
*
**
3 days
14 days
8 weeks
challenge
unimmunized
control vaccine
IL-13 inhibitor vaccine

*(c) ELIspot*




*(d) ICS*

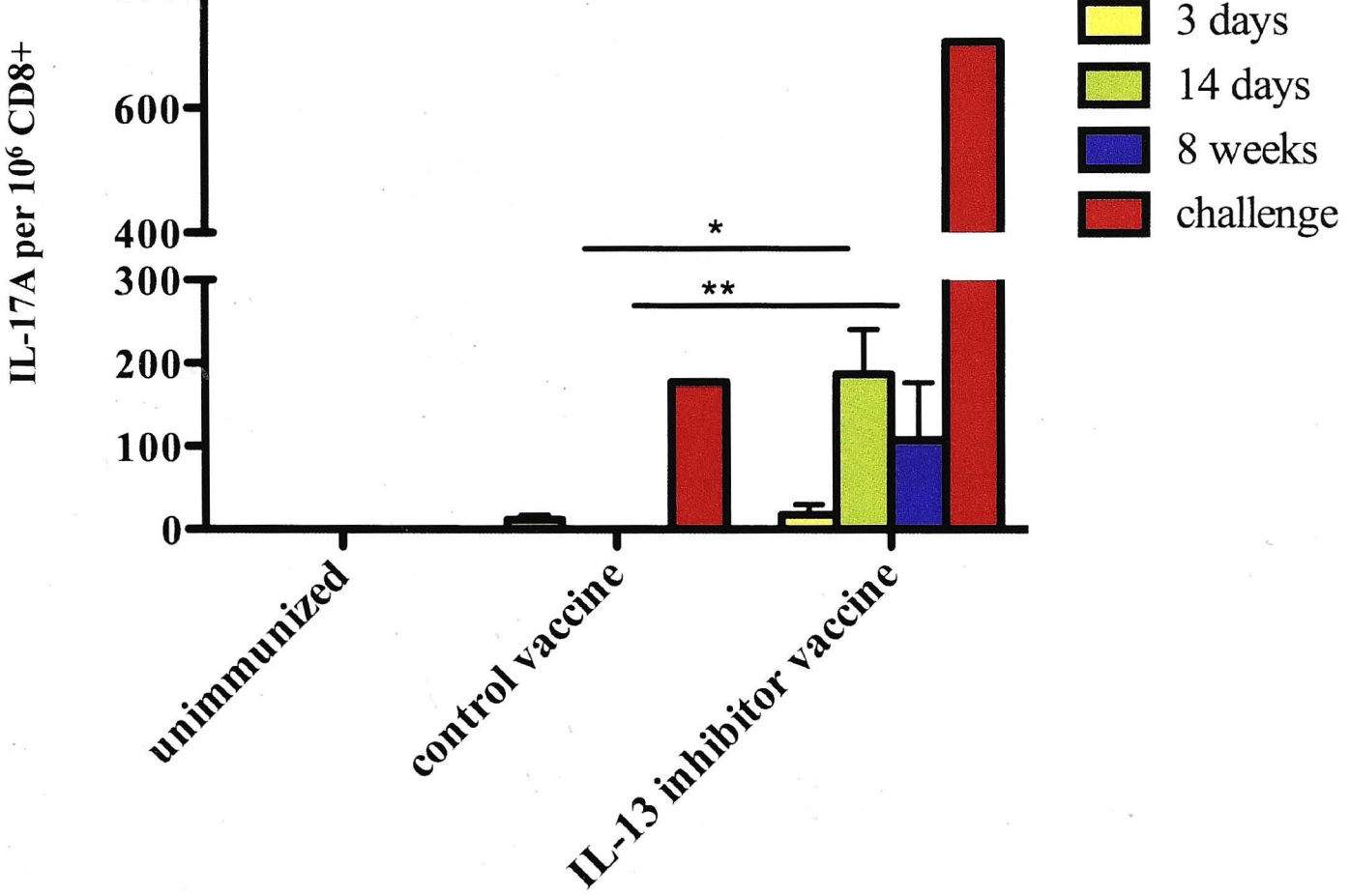
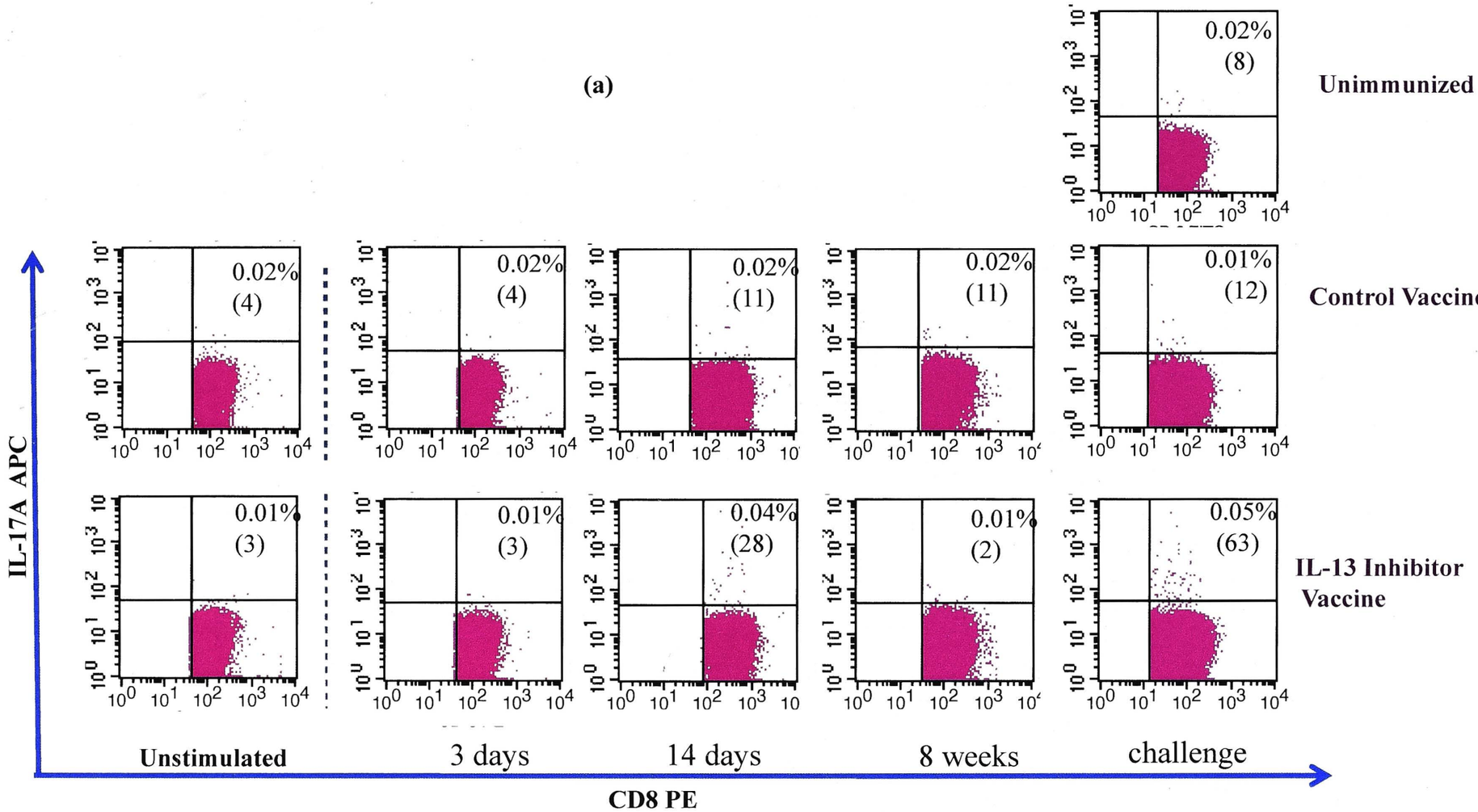


**Fig 5.8: Comparative analysis of IL-17A expression by lung $K^dGag_{197-205}$ specific $CD8^+$ T cells at acute, effector memory and following influenza-HIV challenge**

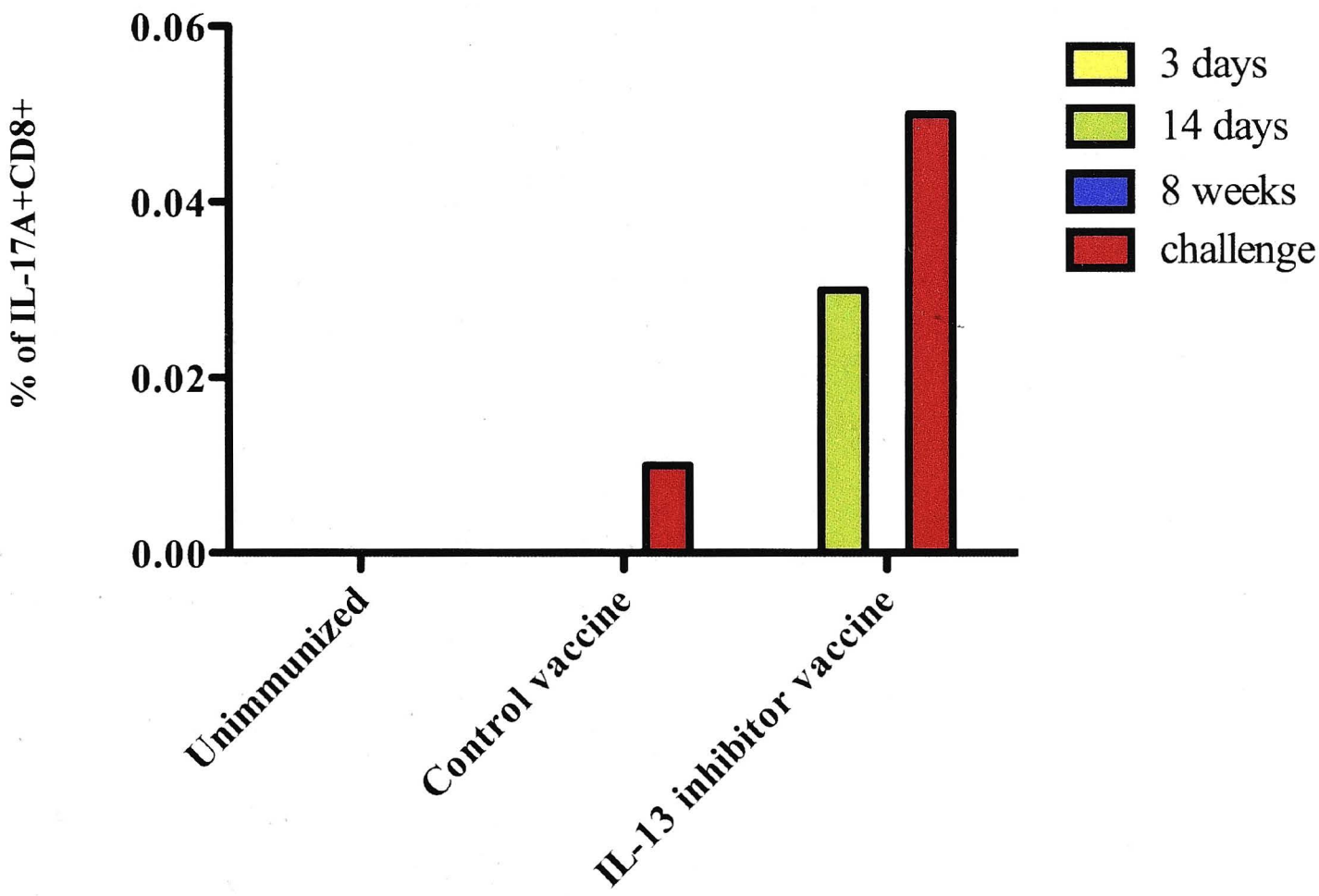
*BALB/c (H-$^{2d}$ Background) mice (n=5-10) were prime-boost immunized with FPV-HIV/VV-HIV (control vaccine) or FPV-HIV IL-13Rα2/VV-HIV IL-13Rα2 (IL-13 inhibitor vaccine). At 3 days, 14 days, 8 weeks and influenza-HIV challenge, lung samples from each group were pooled, harvested and 4 x $10^6$ cells were stimulated for 16-20 hrs with $K^dGag_{197-205}$ peptide and IL-17A expression by $CD8^+$ T cells was measured by intracellular cytokine staining. a) The FACS plots indicate pooled animals from each group. In all FACS plots, the upper right quadrant (R3) indicates R1+R2 and the numbers indicate IL-17A producing $CD8^+$ T cells as a percentage (top) and also the gated number of events (bottom within brackets). b) The graph represents the percentage of $CD8^+$ T cells expressing IL-17A where the unstimulated background value was subtracted. c) The graphs indicate the total number IL-17A SFU per $10^6$ $CD8^+$ T cells measured by ELIspot. Compared to the number of IL-17 cells per $10^6$ $CD8^+$ T cells as measured by ICS. Data are representative of two independent experiments*

*Note: In ICS, Unstimulated values were similar in all experiment time points and the background range was between 0.00-0.02%.*

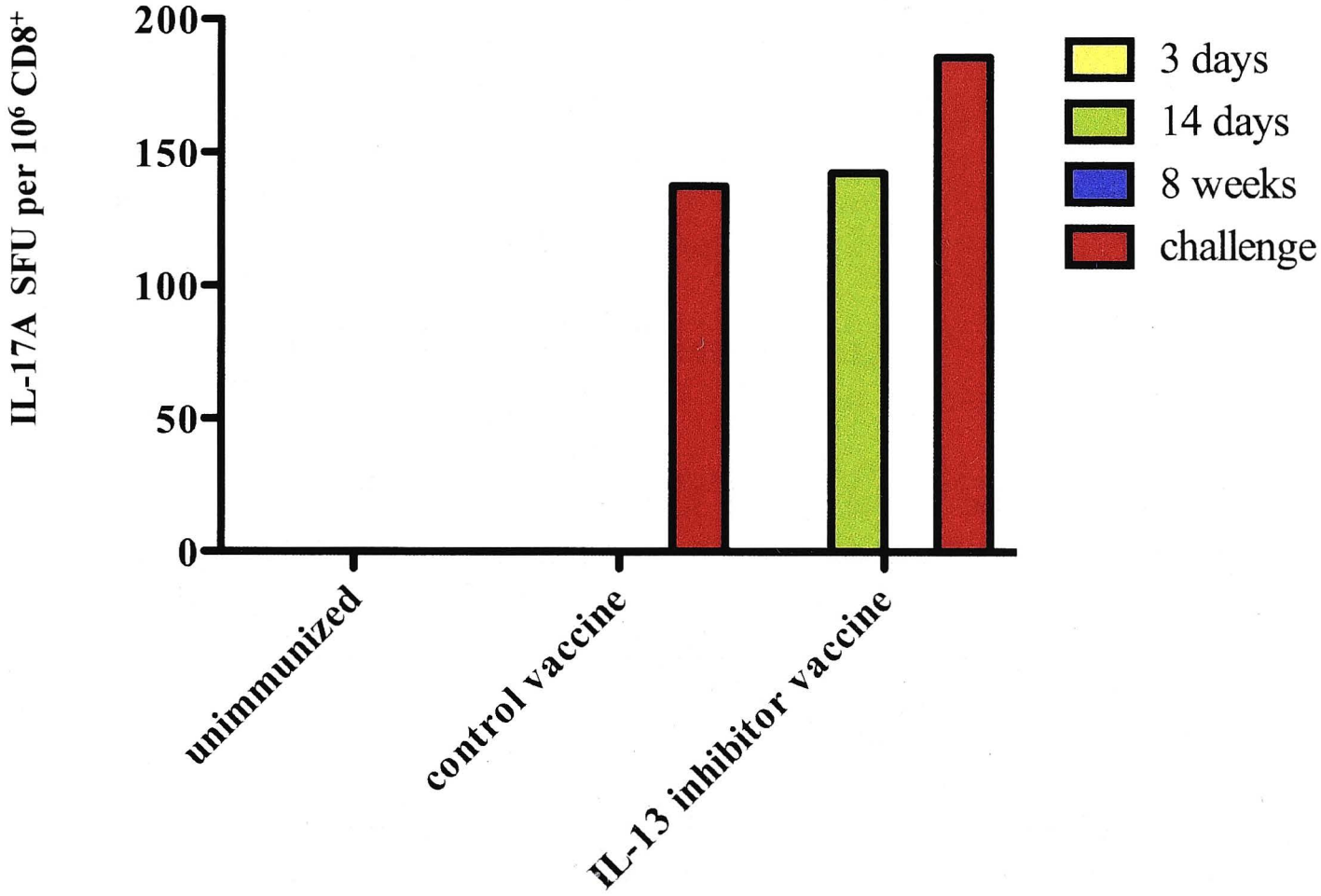
**(a)**


0.02%
(8)
Unimmunized
0.02%
(4)
0.02%
(4)
0.02%
(11)
0.02%
(11)
0.01%
(12)
Control Vaccine
0.01%
(3)
0.01%
(3)
0.04%
(28)
0.01%
(2)
0.05%
(63)
IL-13 Inhibitor Vaccine
IL-17A APC
Unstimulated
3 days
14 days
8 weeks
challenge
CD8 PE

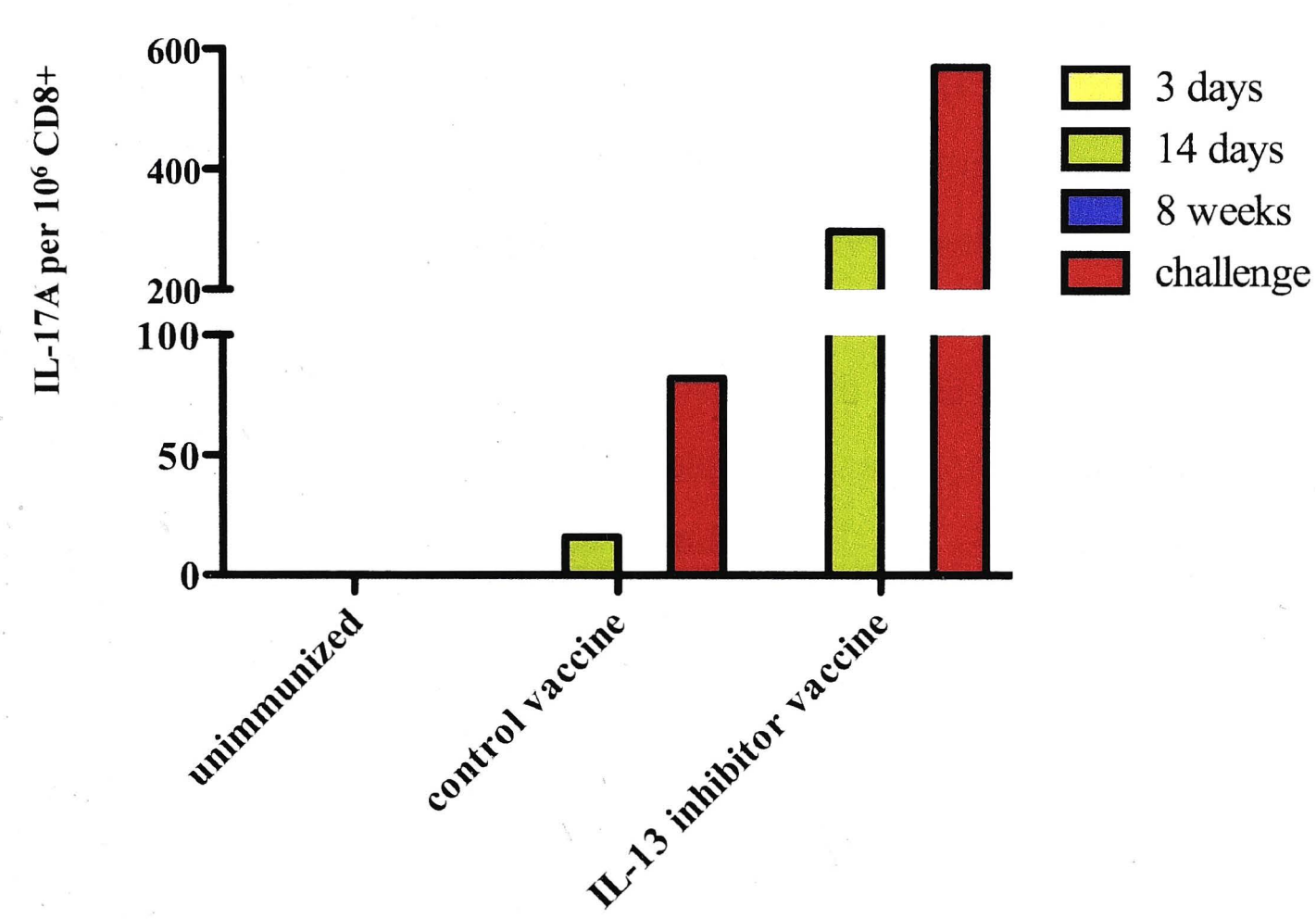
***(b) ICS***


0.06
0.04
0.02
0.00
% of IL-17A+CD8+
3 days
14 days
8 weeks
challenge
Unimmunized
Control vaccine
IL-13 inhibitor vaccine

*(c) ELIspot*




*(d) ICS*



## 5.5 Discussion

Current data obtained from IL-17A ELIspot and ICS clearly indicated that there was a greater IL-17A expression by $K^dGag_{197-205}$ specific $CD8^+$ T cells following IL-13 inhibitor vaccine at effector, memory, and following influenza-HIV challenge compared to the control vaccine and unimmunized controls. However, in IL-13 KO mice, where elevated IL-17A expression was observed (chapter 3), much lower IL-17A expression was detected following IL-13 inhibitor vaccine in BALB/c mice. This can most likely be due to the transient inhibition of IL-13 by this vaccine. In summary, current results clearly indicate that IL-17A expression is significantly modulated by the presence or absence of IL-13 in the cell milieu during vaccination.

Following the novel IL-13 inhibitor vaccine, IL-17A levels were highest at effector stage and following influenza-HIV challenge in both spleen and lung. This suggested that IL-17A expression is mainly regulated at the peak of $CD8^+$ T cell activity. Similarly, influenza-HIV challenge data obtained from IL-13 KO mice and IL-13 inhibitor vaccine have shown enhanced protective immunity (Ranasinghe *et al.* submitted June 2012). Very similar to IL-13-/- mice, these mice showed lower weight loss compared to the mice that received FPV-HIV/VV-HIV (control vaccine). Similarly, IL-17 has shown to play role in vaccine mediated immunity against bacterial infections such as *Helicobacter*, *Bordetella pertussis* and *Pseudomonas aerogenosa*, where its induction by $CD4^+$ T cells correlated with rapid bacterial clearance [32, 42, 193]. Recent studies have shown that vaccination against *Mycobacterium tuberculosis* followed by tuberculosis infection generated IL-23 induced IL-17 production by $CD4^+$ T cells in the lung and induced long lasting memory immunity [21, 39]. Indeed, inclusion of IL-17 as a co-adjuvant in recombinant vaccine against *Eimeria acervulina* has shown to induce protection against the parasite [194]. In the context of viral infections, vaccination against rotavirus generated elevated IL-17 and IFN-γ expression thereby reducing fecal shedding [37, 38]. Interestingly, other studies using IL-17-/- and $STAT3^{CDR}$-/- mice have described that these IL-17A secreting cells were not critical for viral clearance but instead regulated anti-viral immunity through additional mechanism such as neutrophil recruitment [195]. Wang *et al.* have shown that IL-17-/- mice infected with H5N1 influenza exhibited increased weight loss, reduced survival rate high lung pathology [196]. Also, the IL-17A expression by the $CD8^+$ T cells in the lung were associated with protective immunity against influenza virus via the recruitment of neutrophils [19]. The

above observations together with our findings clearly indicate that IL-17A expression can be considered as a marker of protective immunity.

As IL-13 is also involved in the regulation of high avidity CTLs, current data indicate that IL-17A may also play an important role in modulating the avidity of the CTLs and protective immunity. Rahaman *et al.* have shown that IL-13Rα2 could enhance IL-4 mediated STAT3 signaling indirectly [152, 197]. Furthermore, when IL-13 was added at the time or polarization of $CD4^{+}$Th17 cells, there was decrease in the STAT3 phosphorylation [70]. Accordingly, studies have shown that STAT3 plays an important role in the differentiation of $CD4^{+}$ T cells into Th17 cells [198]. This STAT3 mediated differentiation of Th17 cells occurs through induction of IL-23R and transcription factor ROR-γt [199]. Taken together these findings, we can postulate that IL-13 inhibitor vaccine may regulate IL-17A expression by HIV-specific $CD8^{+}$ T cells through the up-regulation of a STAT3 mediated signaling pathway.

In summary, current data clearly indicated that IL-13 inhibitor vaccine can induce enhanced IFN-γ and IL-17A expression by HIV-specific $CD8^{+}$ T cells. Data again substantiate that IFN-γ does not modulate IL-17A expression in HIV-specific $CD8^{+}$ T cells. The greatly elevated IL-17A expression following influenza-HIV challenge suggests that the production of IL-17A by antigen specific $CD8^{+}$ T cells could be a hallmark of protective immunity. Since IL-13 inhibitor vaccine also induced high avidity $CD8^{+}$ T cells, data suggest that IL-17A most likely play a direct or indirect role in $CD8^{+}$ T cell avidity, which is consistent with the findings in chapter 3.

# CHAPTER 6: General Discussion

## General Discussion

Th17 cells, unlike Th1 and Th2 are a newly discovered subset of T cells, which specifically produce range of Th17 family cytokines IL-17A to F [14]. Still there is great debate about the exact role of these Th17 cells and specifically how these cells are regulated under different infection conditions. Majority of the studies in infection models have mainly focused on the role of IL-17A in $CD4^+$ T cells. These studies have shown that IL-17A plays an important role in protective immunity [21, 200, 201]. In the context of HIV/SIV infection, loss of IL-17 production in the intestinal mucosae has been associated with SIV/HIV disease progression in macaques [202, 203]. In HIV prime-boost vaccine model, Ranasinghe *et al.* have shown that IL-4 and IL-13 play a direct role in modulating T cell avidity and protective immunity [165], (Ranasinghe *et al* Submitted). The current study has now shown that IL-4/IL-13 cytokines regulate the IL-17A expression in HIV-specific $CD8^+$ T cells both at the transcriptional and translational level and IL-17A plays an important role in protective immunity. This indicates that IL-17A also plays an indirect role in modulating $CD8^+$ T cell avidity.

Following FPV-HIV/VV-HIV prime-boost immunization, IL-4 KO mice elicited the highest IL-17A expression, which suggested that IL-4 played a more predominant role in down regulation of IL-17A in HIV-specific $CD8^+$ T cells compared to IL-13 KO mice (fig 6.1). In IL-4 KO mice higher levels of TGF-β, IL-6, ROR-γt were also detected suggesting that the IL-17A transcription in HIV-specific $CD8^+$ T cells was mainly regulated in an IL-4 dependent manner. Interestingly, our findings are consistent with allergic asthma studies where IL-4 was shown to be the potent inhibitor of IL-17A expression (Th2 cytokines in particular IL-4 is known to play a pivotal role in allergic asthma and airway hyperresponsiveness) [69]. These studies revealed that the fine balance of IL-4 and IL-17A could determine the severity of asthma [73].

Unlike what was reported in $CD4^+$ T cells [68, 204], current data indicated that IFN-γ did not suppress the IL-17A expression as majority of IL-17A secreting $CD8^+$ T cells were also positive for IFN-γ in KO mice (Fig 6.1). This indicated that in a vaccine setting, IL-17A together with IFN-γ mediates efficient viral clearance in $CD8^+$ T cells.

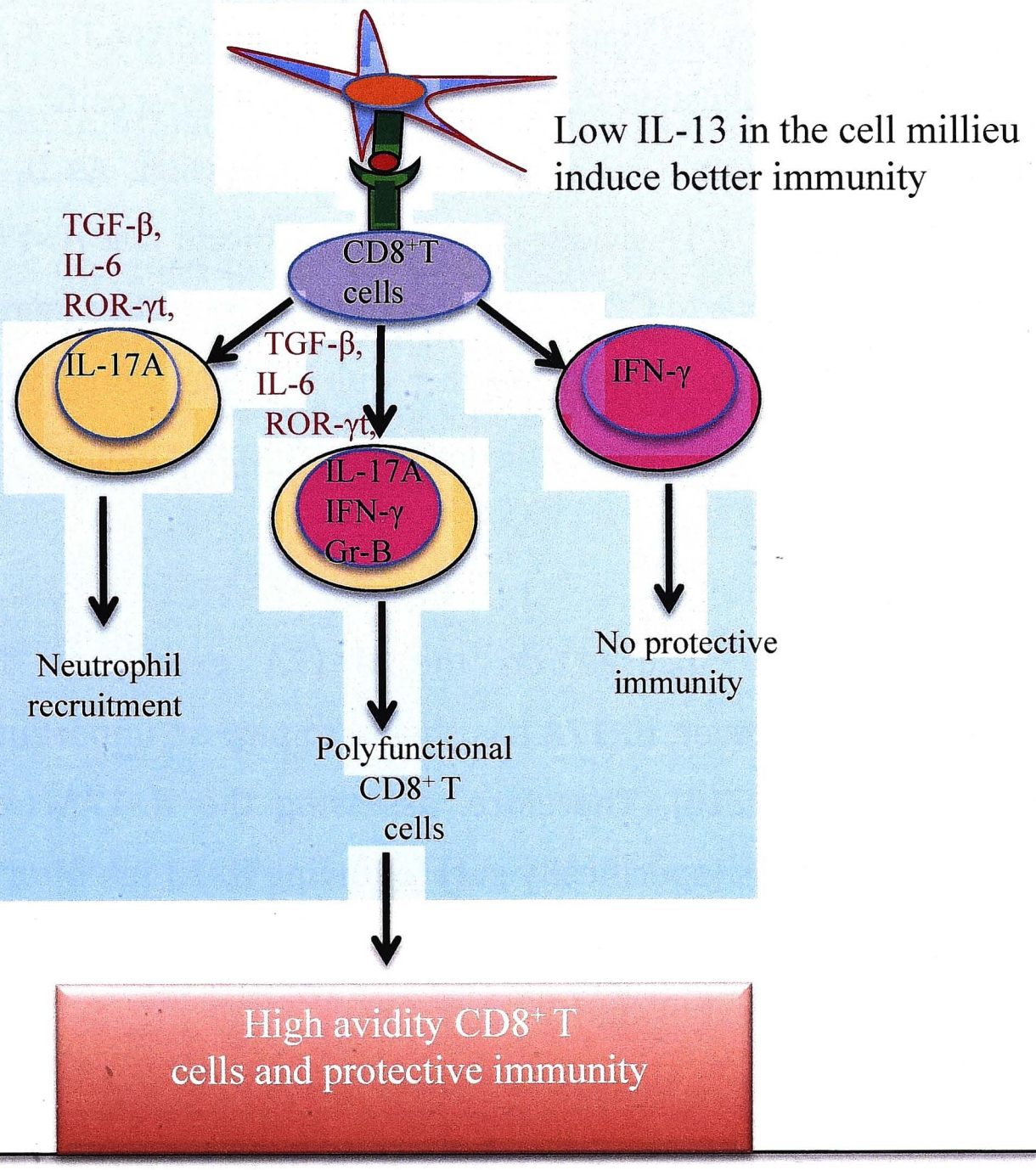
The mRNA studies further substantiated our findings where following peptide stimulation both T-bet (transcription factor for IFN-γ) and IFN-γ were highly up regulated in $CD8^+$ T cells obtained from IL-4-/- mice. Although, studies have shown that ROR-γt was the master transcriptional factor in differentiating/maintaining IL-17A expression, our data indicate that TGF-β plays an important role in the IL-17A induction in $CD8^+$ T cells [172]. Furthermore, current data also showed that IL-23a was not involved in the regulation of IL-17A expression in HIV-specific $CD8^+$ T cells, unlike what has been reported with Th17 $CD4^+$ T cells [205, 206].

Granzyme B is thought to regulate IL-17A expression, as IL-17A expressing $CD8^+$ T cells have shown to be less cytotoxic [181]. Interestingly, in the current study, HIV-specific $CD8^+$ T cells obtained from IL-13 KO mice produced the highest granzyme B and showed reduced IL-17A expression whilst the IL-4 KO mice showed the highest IL-17A expression but lower granzyme B. These observations were consistent with previous findings in our laboratory where IL-13 KO mice were found to express elevated granzyme B by HIV-specific $CD8^+$ T cells compared to the other KO groups tested (fig 6.1) (Ranasinghe 2007). The IL-13 KO mice showed enhanced granzyme B, moderate IL-17A, and IFN-γ expression by HIV-specific $CD8^+$ T cell. These results correlated well with our previous avidity studies, where FPV-HIV/VV-HIV vaccination of IL-13 KO mice showed high avidity effector and memory HIV-specific $CD8^+$ T cells with better protective immunity following a surrogate HIV challenge compared to IL-4 KO mice (Ranasinghe 2009, Ranasinghe Submitted).

The new IL-13 inhibitor vaccine (FPV-HIV IL-13Rα2/VVHIV-IL-13Rα2), also induced higher IL-17A expressions in $CD8^+$ T cells compared to the control vaccine (FPV-HIV/VV-HIV), and were found to be better protective following a mucosal challenge (Ranasinghe *et al* submitted). However, compared to IL-13 KO mice, the WT BALB/c mice that received the IL-13 inhibitor vaccine were less protective and showed lower numbers of IL-17A expressing HIV-specific $CD8^+$ T cells (Ranasinghe et al submitted). This is not entirely surprising as unlike IL-13 KO mice where a complete lack of IL-13 was found in the cell milieu, the novel IL-13Rα2 vaccine only inhibited IL-13 activity in a transient manner at the vaccination site. The current observations clearly indicate that IL-13 acts upon IL-17 and these two cytokines plays an important role in modulating $CD8^+$ T cell avidity and protective immunity in mice. Even though some, studies contradict our findings for example, during respiratory syncytial virus infection

**Fig 6.1 Schematic diagram depicting IL-17A mediated protective immunity**

**PRIME-BOOST :FPV-HIVIL-13Rα2/VV-HIV IL-13Rα2**



elevated IL-17A expression was reported to inhibit $CD8^+$ T cell effector function and increase viral proteins [207], majority of the studies have shown that elevated IL-17A correlated with protective immunity. For example, elevated IL-17 and IFN-γ expression following rotavirus vaccination was associated with reduced shedding of the virus [37, 38]. Similarly, $CD8^+$ T cells producing IL-17A in the lung were involved in protective immunity during influenza infection (Hamada et al., 2009). Therefore, our studies indicate that the activity of IL-17A largely depends upon presence or absence of IL-4/IL-13 in the cell milieu and this can have a major impact on $CD8^+$ T cell cytotoxicity and protective immunity (Fig 6.2).

Collectively, data in this thesis clearly demonstrate that i) both IL-4 and IL-13 modulate the expression of IL-17A by HIV-specific $CD8^+$ T cells, where IL-4 play a more predominant role in this modulation than IL-13 and ii) in HIV-specific $CD8^+$ T cells, TGF-β, IL-6 and ROR-γt mainly regulate the IL-7A expression. As IL-4 and IL-13 significantly modulate $CD8^+$ CTL avidity, results also indicate that IL-17A is also an indirect player in the regulation of $CD8^+$ T cells avidity and protective immunity. These finding suggest that in a vaccine context, together with IFN-γ and Granzyme B, IL-17A also can be used as a marker of effective cell mediated protective immunity.

**Future directions**

- This study mainly focused on the IL-17A expression in systemic compartment. However, IL-17A has shown to play an important role in the gut mucosae [208-210]. Therefore, assessing the IL-17A expression in mucosal $CD8^+$ T cells (specifically gut) following IL-13 inhibitor vaccination would provide valuable insights into how IL-17A is regulated at the mucoase in the context of an HIV-1 vaccine.

- In this study IL-4 was the main inhibitor of IL-17A expression. Therefore, creating an HIV-1 vaccine that could inhibit both IL-4 and IL-13 has great potential to induce enhanced IL-17A and enhanced cytotoxicity, which may offer better protective immunity.

- Also previous studies in our laboratory have shown that route of vaccine delivery can modulate IL-4 and IL-13 expression. Specifically, mucosal immunization induces low IL-4/IL-13 expression by HIV-specific $CD8^+$ T

cells compared to systemic delivery. Therefore, following systemic and mucosal vaccine delivery assessing the IL-17 expression will also provide useful information on the importance of designing mucosal vaccines for HIV-1.

- IL-25 or IL-17E unlike IL-17A belongs to the newest member of IL-17 family and mediates Th2 immune response by inhibiting Th1 and Th17 response [78-81, 211]. Studies have shown that neutralization of IL-25 in an asthma model lead to the down regulation of IL-13 expression and a reciprocal increase in IL-17A correlating to protective immunity against airway hyper responsiveness [212]. Therefore, future studies evaluating the role of IL-25 and IL-17A regulation in HIV-specific $CD8^+$ T cells may provide valuable information on how T cell avidity is modulated *in-vivo*.

- Studies designed to further understand the mechanisms by which IL-17A induce better cell mediated protective immunity will aid in the constructions of more effective future vaccines not only against HIV-1 but also for other chronic diseases.

**Fig 6.2 Schematic diagram summarizing the expression of IFN-γ, IL-17A and granzyme-B and $CD8^+$ T cell avidity following prime-boost immunization**

## PRIME-BOOST :FPV-HIV/VV-HIV

| | BALB/c | IL-4-/- | IL-13-/- | STAT6-/- |
|---|---|---|---|---|
| **IFN-γ** | +++ | ++++ | +++ | +++ |
| **IL-17A** | - | ++++ | +++ | +++ |
| **Gr-B** | + | ++ | ++++ | ++ |
| **Avidity** | ++ | +++ | ++++ | +++ |

# REFERENCES

## References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